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Maladaptive plasticity in anorexia nervosa: evidence of a periphery-to-brain crosstalk in a preclinical model

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Francesca Mottarlini

Supervisor: Prof. Fabio Fumagalli

Coordinator: Prof. Giuseppe Danilo Norata

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Riassunto

L'anoressia nervosa (AN) è una patologia psichiatrica severa e complessa che, ad oggi, vanta il più elevato tasso di mortalità e ricaduta tra le patologie psichiatriche. Essa colpisce principalmente le giovani donne in età puberale, con un tasso di incidenza sempre in crescita tra gli adolescenti. La patologia insorge prevalentemente in soggetti vulnerabili, caratterizzati da una costante paura di aumentare di peso, i quali, per contrastarla, mettono in atto comportamenti scorretti come l'auto-imposizione di una dieta fortemente restrittiva e ipocalorica in combinazione, spesso, con esercizio fisico eccessivo. La principale conseguenza di questi comportamenti aberranti, esasperati da una concezione distorta della propria forma fisica, è una severa riduzione di peso corporeo, che porta il soggetto verso una condizione di pericolosa emaciazione, nella maggior parte dei casi fatale. Data la complessità di questo disturbo, la molteplicità dei fattori di rischio neurobiologici predisponenti e la mancanza di ricerca preclinica, l'eziopatogenesi dell'AN è ancora sconosciuta e attualmente non esiste alcuna terapia farmacologica efficace.

L'ipotesi più accreditata riguardo i meccanismi implicati nell'insorgenza e nel mantenimento dei comportamenti aberranti legati all'AN ipotizza un'alterazione a livello del sistema nervoso centrale dei neurocircuiti che controllano i processi motivazionali. Lo sbilanciamento dei meccanismi di ricompensa e di inibizione, in combinazione con alterazioni nei modulatori dell'appetito, è stato proposto essere in grado di indurre un'alterata risposta a stimoli salienti, naturalmente piacevoli, come il cibo. Tali risposte aberranti diventano quindi rinforzi positivi e promuovono l'instaurarsi di un circolo vizioso rendendo estremamente appagante la capacità di esercitare un completo controllo sul cibo e sulla propria forma fisica. A livello neurobiologico, un numero crescente di evidenze, sia nell'uomo che in modelli animali, riporta che la condizione indotta dall'AN altera i livelli di espressione e rilascio di neuropeptidi come grelina, leptina, del fattore di rilascio della corticotropina e degli endocannabinoidi sia a livello centrale che sistemico. Inoltre, è stato dimostrato che l'AN causa alterazioni nei livelli degli ormoni circolanti, nella funzionalità degli organi periferici e nei tessuti metabolicamente attivi come il muscolo scheletrico, suggerendo un potenziale collegamento tra segnali periferici e cervello.

L'obiettivo di questo progetto di tesi è stato, quindi, quello di investigare dal punto di vista preclinico i meccanismi neurobiologici alla base della patologia, ponendo l'attenzione sul coinvolgimento degli organi periferici nell'instaurare un asse di comunicazione

neurometabolico periferia-cervello, con lo scopo ultimo di individuare marcatori molecolari coinvolti nel mantenimento del fenotipo anoressico.

A tale scopo, è stato impiegato uno dei modelli animali di AN più accreditati e condivisi dalla letteratura scientifica: il modello *Activity-based anorexia* (ABA), che comporta l'esposizione di ratte femmine adolescenti ad un regime di alimentazione limitato e libera attività di corsa su una ruota meccanica. In queste condizioni abbiamo dimostrato, in linea con i dati di letteratura, che i roditori esposti al modello ABA riducono l'assunzione di cibo e in parallelo incrementano l'attività di corsa sulla ruota, subendo di conseguenza una severa perdita di peso corporeo, riproducendo così i sintomi principali dell'AN.

Studi clinici di *neuroimaging* condotti su individui affetti da AN hanno evidenziato alterazioni a livello metabolico, morfologico e di connettività funzionale, soprattutto nelle regioni frontali, temporali e talamocorticali. Inoltre, è stato dimostrato che tali alterazioni sono correlate a risposte aberranti a stimoli naturalmente piacevoli, come il cibo, e a deficit cognitivi nei processi di memoria e attenzione. Sulla base di tali evidenze in campo clinico, a seguito della caratterizzazione comportamentale del modello ABA, sono state condotte analisi molecolari di espressione genica e proteica, analisi strutturali e morfologiche delle arborizzazioni dendritiche e test cognitivo-comportamentali. Il paradigma sperimentale ha previsto che tutte le analisi molecolari e i test comportamentali siano stati effettuati a due tempi di sacrificio: al giorno postnatale (PND) 42, corrispondente alla fase acuta della patologia, e dopo sette giorni di recupero del peso corporeo, al PND49, al fine di valutare sia le alterazioni provocate dall'induzione del fenotipo che le alterazioni persistenti nonostante il recupero del peso corporeo. Inoltre, è stato valutato, oltre che l'impatto dell'induzione del fenotipo anoressico mediante il modello ABA, anche il contributo delle singole condizioni sperimentali, ovvero restrizione alimentare ed esercizio fisico.

Tali analisi hanno riguardato i principali marcatori molecolari della sinapsi glutammatergica nel Nucleus Accumbens (NAc) e nella corteccia mediale prefrontale (mPFC), due delle principali aree del circuito della ricompensa deputate alla regolazione dei processi motivazionali ed esecutivi. Inoltre, sulla base dell'ipotesi che lo squilibrio energetico, attraverso un'alterata regolazione di molecole presenti in organi periferici, possa portare all'alterazione di neurocircuiti responsabili dei processi motivazionali e cognitivi, è stata condotta un'analisi neurometabolica del pathway PGC1α/FNDC5/Irisin/BDNF correlando alterazioni molecolari a livello del muscolo scheletrico con alterazioni osservate

nell'ippocampo (Hip), rispettivamente coinvolti nell'attività fisica e nei processi di memoria e apprendimento.

I risultati ottenuti, mediante studi di imaging strutturale e di espressione proteica in diverse frazioni subcellulari, mostrano che l'esposizione alla combinazione di restrizione alimentare ed esercizio fisico altera l'omeostasi della sinapsi glutammatergica nella mPFC e nel NAc. Tali alterazioni che coinvolgono sia la composizione recettoriale che la struttura della sinapsi glutammatergica si osservano al raggiungimento del fenotipo anoressico, effetti che persistono nonostante il periodo di recupero del peso corporeo. Inoltre, tali alterazioni suggeriscono che possano contribuire a spiegare il deficit della memoria temporale osservata negli animali ABA esposti a test cognitivi specifici. Per quanto riguarda l'asse neurometabolico PGC1α/FNDC5/Irisin/BDNF, abbiamo osservato un'iperattivazione dell'asse a seguito dell'induzione del fenotipo anoressico, data dall'incremento dei livelli di espressione proteica di PGC1α e FNDC5 nel muscolo soleus e dell'aumento dei livelli circolanti della miochina Irisin. L'effetto dell'iperattivazione dell'asse in fase acuta induce a livello centrale, in Hip, un'alterazione della neurotrofina BDNF, come dimostrato dalla riduzione della sua espressione proteica e delle vie di segnalazione intracellulare da essa controllate specificatamente negli animali ABA. Anche in questo caso abbiamo osservato che le alterazioni sono persistenti nonostante il recupero del peso corporeo, poiché, dopo sette giorni di remissione, in Hip permane la riduzione dell'espressione di BDNF, suggerendo un possibile ruolo mal-adattivo della neurotrofina nei processi di apprendimento legati alla ricompensa.

In conclusione, tali risultati suggeriscono che le alterazioni osservate possano essere in grado di ridurre la capacità delle ratte ABA di rispondere a stimoli esterni in maniera adeguata e di perpetuare i comportamenti aberranti tipici del circolo vizioso dell'AN. In particolare, la connessione fra alterazioni molecolari negli organi periferici, e disfunzioni nella plasticità cerebrale, può almeno parzialmente, contribuire ad alterare l'omeostasi energetica ed essere responsabili non solo del mantenimento del fenotipo anoressico, ma anche della vulnerabilità alla base dei fenomeni di ricaduta.

Abstract

Anorexia Nervosa (AN) is a devastating psychiatric disorder affecting pubescent females ninefold more than males, in a period of life extremely vulnerable to external stimuli: the adolescence. AN begins with a self-induced restrictive diet to lose weight, below 85% of expected body mass index, that in combination with intense physical activity and aberrant body image concern, it progresses to an *out-of-control* spiral. In this condition, the positive experience of control on food intake and intense exercise is indeed extremely rewarding for the patient, reinforcing the dieting behavior. AN is associated with the highest mortality rate among the wide family of psychiatric disorders, and despite the knowledge about the clinical symptomatology, AN etiopathogenesis remains unclear, treatment is challenging and often hampered by high relapse. At the neurobiological level, patients suffering from AN display altered neural activity, morphological and functional connectivity in the fronto-striatal and thalamo-cortical circuits. In particular, hypoglutamatergic transmission and aberrant excitability of cortical and striatal regions observed in AN patients might underpin cognitive deficits that fuel the vicious cycle of dieting behavior. It has been suggested that the driving force of such abnormal behavior might be represented by an altered balance between reward and inhibition mechanisms in the brain, the first mainly mediated by the Nucleus Accumbens (NAc), and the latter being normally triggered by the medial prefrontal cortex (mPFC) that is immature and still growing during adolescence. Such dysfunctional mechanisms combined with alterations in the levels of appetite modulators, such as ghrelin and leptin, and dysregulations in peripheral organs and metabolic active tissues functionality may lead to a distorted response to salient stimuli, such as food, fueling the maintenance of the anorexic phenotype. AN is not only a brain disorder but it is rather a systemic disease that affects the whole body, particularly skeletal muscles and circulating hormones, suggesting the presence of a crosstalk between peripheral signals and brain functions.

Therefore, the main goal of this project is to investigate from a preclinical point of view the neurobiological mechanisms involved in the pathophysiology of AN. We pointed our attention to the possibility that the combination of food restriction with intense exercise, the hallmarks of AN, activates dysfunctional periphery-to-brain crosstalk that may drive for weight loss seeking inducing aberrant dieting behaviors, affecting reward processes and cognition, via altered neurometabolic pathways in specific areas of the brain, and in turn, promoting the long-lasting maintenance of AN disorder.

Therefore, with the aim to investigate the central and peripheral alterations in biological mechanisms that may support the anorexic condition and induce cognitive impairments, we employed the well-known animal model of AN, the activity-based anorexia (ABA) rat model, which recapitulates human AN by combining caloric restriction and physical exercise to induce self-induced body-weight loss. We have demonstrated that, under a paradigm of food restriction and free access to a mechanical activity wheel, adolescent female rats showed reduced food intake, reduced body weight and increased running activity on the wheel, thus developing the anorexic phenotype.

To dissect the neurobiological underpinnings of this condition and the cognitive-related dysfunctions during a critical developmental period, we performed structural, morphological and molecular studies on critical brain areas of the reward system, crucially involved in processing feeding information and in mediating high-level cognitive functions such as the NAc and the mPFC. In both AN patients and animal models of AN, evidence exists of glutamate homeostasis dysfunctions, which has been proposed as a signal of altered processing of food reward. Thus, we firstly focused our attention on critical determinants of the glutamatergic synapse evaluating AMPA, NMDA receptors, their scaffolding proteins and their localization at the post-synaptic density, dendritic spines density and morphology, and lastly cognitive-related outcomes. Furthermore, based on the hypothesis that the energy imbalance, via altered regulation of molecules released by peripheral organs, can lead to the alteration of neurocircuits responsible for motivational and cognitive processes, we performed a neurometabolic analysis of the PGC1α/FNDC5/Irisin/BDNF pathway, linking molecular alterations in skeletal muscle with alterations in the hippocampus (Hip), respectively involved in physical activity and in memory and learning processes. All the molecular analysis has been performed at two time points: early after the induction of the anorexic phenotype at post-natal day (PND) 42, which mimics the acute phase of the pathology, and after a period of body weight recovery at PND49, in order to investigate both the alterations induced by the AN acute phase and the potential long-lasting alterations that persist even after the body weight recovery period.

Overall, our analysis revealed that the combination of a restricted regimen of food intake and free wheel access induced a general dysregulation of the glutamatergic homeostasis in both NAc and mPFC regions. We observed AMPA and NMDA receptor subunit reorganization and retention in different subcellular fractions, paralleled with structural changes in dendritic spines density and morphology and altered temporal memory performance. Of note, all these

alterations persist even after the body weight recovery. Interestingly, the analysis of the neurometabolic axis on the PGC1α/FNDC5/Irisin/BDNF pathway revealed an initial hyperactivation of the axis induced by the induction of the acute phase of AN, as shown by increased PGC1α and FNDC5 protein levels in muscle tissues and increased irisin plasma levels observed at PND42. In the Hip, BDNF transcription was increased, while protein translation was reduced specifically in ABA rats. After body weight recovery, despite PGC1α and FNDC5 protein levels were normalized, BDNF expression in the Hip was persistently reduced, suggesting its putative involvement, not only in altering the hippocampal neuroplasticity, but also in the induction of associative reward learning alterations that may underpins the observed long-lasting memory deficits.

In conclusion, the obtained results point toward the reorganization of the glutamatergic synapse structure and composition in crucial brain areas of the reward system, as critical factor in driving the motivational mechanisms that fuel the maladaptive behaviors underlying AN. Moreover, our data provides novel insights in the involvement of a periphery-to-brain neurometabolic crosstalk even when body weight is restored. These molecular determinants of maladaptive plasticity could represent a signal of altered processing of food reward, and a vulnerability trait for relapse. Moreover, the herein shown AN-induced dysregulation of the glutamatergic system and hippocampal BDNF system coupled with an altered metabolic profile might be the trigger that could lead, in turn, to cognitive dysfunction, which is consistently observed in AN patients.

1 Introduction

"The want of appetite is, I believe, due to a morbid mental state...I prefer---the more general term [anorexia] 'nervosa', since the disease occurs in males as well as females, and is probably rather central than peripheral."

Sir William Gull, 1874 (Transactions of the Clinical Society of London 7: 22–28)
From The Lancet Psychiatry 2015

Sir William Gull was the first physician who reported a case of Anorexia Nervosa in London, in the Lancet in 1888 (Silverman, 1997), defining for the first time the pathological condition of *neurotic loss of appetite* as Anorexia Nervosa.

1.1 *Anorexia nervosa*

Anorexia Nervosa (AN) is a serious life-threatening psychiatric disorder dominated by a constant fear of gaining weight and by the absolute control over the body shape and food intake (DSM-V, 2013). This way of reasoning brings AN patients to apply a restricted diet, dramatically reducing daily food consumption, and to perform strenuous exercise regimens of activity to fulfil the desire of lose weight. In most of the cases, body weight (BW) drops below 85% of expected body mass index (BMI), as stated by the Diagnostic and Statistical Manual of Mental Disorders (DSM-V, 2013). Indeed, the core symptoms of AN are reduced food intake, strenuous hyperactivity, that as consequence, cause more than 25% loss of weight in respect to their physiological BMI. All these symptoms are surrounded and sustained by psychological implications and personality predispositions that in most of the cases contribute to the onset of the disease and to its exacerbation. Patients affected by AN display perfectionism expressed in setting high-performance standards and overcontrol, depression and low self-esteem, the feeling of self-ineffectiveness which causes isolation and loneliness, difficulties in building relationships and in trusting people, and low interoceptive awareness resulting from a difficulty of recognizing emotional states and signals arising from self-body experience (Aguera et al., 2019; Skowron et al., 2020). Nonetheless, the impact of socio-cultural imposed standards on the body image concern, such as slim body and body dissatisfaction shaped by mass media, and the pressure associated to reach ideal appearances have been indicated as key factors to the current food restriction emergency and cannot be underestimated (Holland & Tiggemann, 2016; Holland & Tiggemann, 2017).

AN belongs to the class of eating disorders (ED) and it is characterized by high rates of chronicity, morbidity, and mortality. AN predominantly affects pubescent females, 9 times more than males (Aoki et al., 2017) with a world-wide incidence rate in the general population around 1-2% (Hudson et al., 2007; Smink et al., 2014). AN has the highest mortality rate among all psychiatric diseases (Arcelus et al., 2011), that is 200 times greater than the suicide's rate (Aoki et al., 2017; Sullivan, 1995). The lifetime prevalence of AN was found to be 0.3% among adolescents aged 13–18 years in USA (Swanson et al., 2011) and 1–4% among girls aged 12–18 years in Europe (Herpertz-Dahlmann, 2015). Of note, recent studies report incidence rates of 100–200/100,000 person/years in young females (15–19 years), which is comparable to, for example, the incidence of diabetes type 1 (Javaras et al., 2015; Seitz et al., 2019; Smink et al., 2016). About 25% of the individuals affected by AN suffer from a chronic and relapsing course

of the disease: 20% of them remain chronically ill and up to 70% develop psychiatric comorbidities (Steinhausen, 2002), including strong associations with mood and anxiety disorders, personality disorders, self-harm and suicidality, as well as substance use disorders (Kask et al., 2016; Moskowitz & Weiselberg, 2017). What it is alarming is that the number of hospitalized children and adolescents has increased (Holland et al., 2016) and the age of onset has decreased.

Besides epidemiological studies that have explored different sampling strategies to register incidence and prevalence rates of AN in the world, we have to consider that numbers are limited and restricted to community studies, and not to the treatment-seeking samples, due to the fact that the majority of individuals do not access treatment, primarily because they do not recognize the disease, and thus refuse any therapy (Treasure et al., 2015). In general, the incidence rates from all the above-mentioned studies are comparable and with good reasons suggest that the rate of first diagnosis of AN is highest among young adolescent individuals, 15–20 years of age. Subjects with AN experience disturbance in their body image concern: especially many teenagers figure a complete distorted picture of their body shape, perceiving them as overweighted even if they are pathologically underweighted and malnourished. All these feelings are owing, at least in part, to the current imposed socio-cultural model of beauty, but are further complicated by the fragile personality traits, low self-esteem, and irrational behaviors typical of the adolescence period, that is indeed one of the most vulnerable and critical windows of the human being life (Dow-Edwards et al., 2019; Larsen & Luna, 2018). Along this line of reasoning, food avoidance, BW control and physical activity become obsessions, and bring individuals in the vicious loop of AN.

Despite the negatives, and differently from other psychiatric conditions, whose onset at an early age leads to a severe course of the disease, adolescents with AN have a better prognosis than adults. Accordingly, some studies have shown that after one year of psychosocial therapies about 75% of adolescent patients are in total or partial remission (Lock et al., 2010; Watson & Bulik, 2013). This does not exclude the fact that patients still manifest anorectic-related pathological traits, such as obsessive-compulsive behaviors, perfectionisms and overcontrol, that compromise their full recovery and enhance the risk of relapse.

As presented below, Treasure and colleagues propose a classification of the different stages of AN based on the severity of behavioral and psychobiological traits experienced by the patients during the course of the disease (Treasure et al., 2015). People presenting feeding problems, low BMI coupled with cognitive rigidity and reduced reward pleasure to salient stimuli are at high risk to develop AN, that if persist over time progress towards the full syndrome, characterized by the classical symptoms of AN. Moreover, if not properly diagnosed and treated in time it might culminate in a severe enduring pathology with strong rooted traits, such as social isolation, habits, and impaired life quality (Fig 1). AN persistence is considered the tendency of AN patients to follow a protracted self-starvation behavior even without a sufficient amount of energy to sustain that condition over time. This behavior is probably driven, during onset of the disease, to goal directed behaviors that then shift towards habits, and settle in the patient's life (Walsh, 2013).

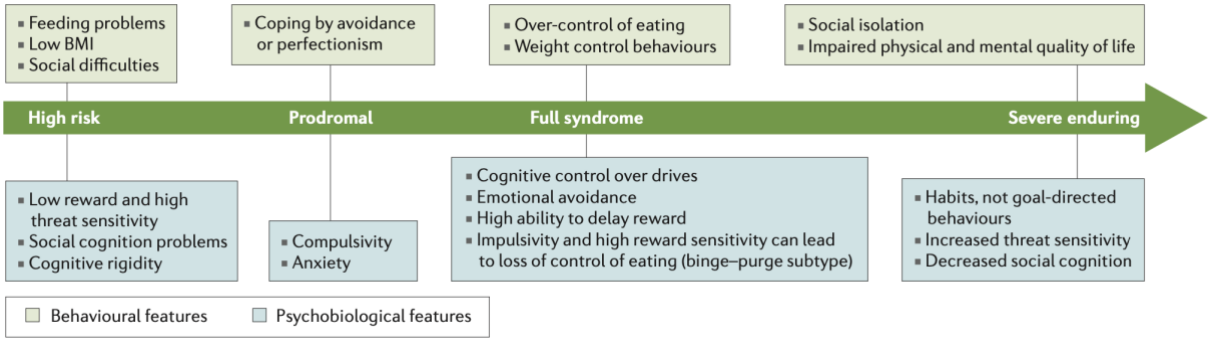

Figure 1 *Behavioral and psychobiological features of anorexia nervosa across the various stages of the disorder* (Treasure et al., 2015).

Although progress has been made in identifying psychological, developmental, or genetic and neurobiological factors that might play a role in the onset of the disorder, the etiopathogenesis of AN still unknown and the lack of preclinical research remains critical. In this scenario, AN emerges as a complex multifaced disorder that, akin to other psychiatric pathologies, has multifactorial origins and represents a real social and clinical burden.

1.2 *Diagnostic criteria, clinical implications, and treatments*

Restriction of energy intake relative to requirements (1), intense fear of gaining weight or of becoming fat (2), disturbances in the body image concern (3), BW less than the lowest weight considered normal based on age and sex (4) and refusal to maintain a normal BW (5) are the main diagnostic criteria used by clinicians to diagnose AN, which are expressed in the revised and most up-to-date version of the Diagnostic and statistical manual of mental disorders released by the American Psychiatric Association (APA) in 2013 (DSM-V, 2013). Over time the diagnostic criteria of AN have been refined and evolved in order to clarify the language and eliminate possible misunderstanding and thus, reduce the risk of diagnosis overestimation (Call et al., 2013). Briefly, in the DSM III, the weight loss criterion was restricted to a 25% weight loss, with the specification that the weight loss had not to be due to other medical pathologies, and the amenorrhea was not considered as a valuable criterion at that time (DSM-III, 1980). In the subsequent DSM III-R, that preceded the approved DSM-IV the weight criterion was changed to a 15% weight loss and the amenorrhea reintegrated as a diagnostic criterion (DSM-IV, 1990). Then, in the last DSM-V version, based on conflicts with inclusion of male individuals, women who use exogenous hormones, and in particular because of adolescents who have not yet reached menarche, the amenorrhea was definitely excluded from the diagnostic criteria and considered as no more relevant for AN diagnosis (DSM-V, 2013) (Tab 1).

The APA classified the severity of anorexia nervosa along four levels by use of the individual's BMI and stated: mild (BMI ≥17 kg/m^2), moderate (BMI 16–16·99 kg/m^2), severe (BMI 15–15.99 kg/m^2), and extreme (BMI <15 kg/m^2). Despite clinicians have all the competences and the guidelines, and even though the symptomatology of AN is well-defined and clear, there are many different risk factors that interact and contribute to the disease susceptibility and affect the quality of life of the patients (Fig 2).

Furthermore, patients that suffer from such eating disorder are characterized by the persistent lack of recognition of the seriousness of their current condition, especially of the dramatic low body weight, thus to elaborate a correct diagnosis get highly more complicated. Accordingly, AN is recognized as an *ego-syntonic* disorder because patients experience their symptoms as congruent with their own values and they will not do anything to go against the proper belief (Vitousek et al., 1998).

	DSM-IV		DSM-5[2]
A	A refusal to maintain bodyweight at or above a minimally normal weight for age and height (eg, weight loss leading to a maintenance of bodyweight less than 85% of that expected, or failure to make expected weight gain during period of growth, leading to bodyweight less than 85% of that expected).	A	Restriction of energy intake relative to requirements, leading to a significantly low bodyweight in the context of age, sex, developmental trajectory, and physical health. Significantly low weight is defined as a weight that is less than minimally normal or, for children and adolescents, less than that minimally expected.
B	Intense fear of gaining weight or becoming fat, even though underweight.	B	Intense fear of gaining weight or of becoming fat, or persistent behaviour that interferes with weight gain, even though at a significantly low weight.
C	Disturbance in the way in which one's bodyweight or shape is experienced, undue influence of bodyweight or shape on self-evaluation, or denial of the seriousness of the current low bodyweight.	C	Disturbance in the way one's bodyweight or shape is experienced, undue influence of body shape and weight on self-evaluation, or persistent lack of recognition of the seriousness of the current low bodyweight.
D	In postmenarcheal females, amenorrhoea—ie, the absence of at least three or more consecutive menstrual cycles. (A woman is considered to have amenorrhoea if her periods occur only following hormone—eg, oestrogen administration).	D	..

Table 1 *Comparison of diagnostic criteria for anorexia nervosa according to DSM-IV versus DSM-V* (Zipfel et al., 2015)

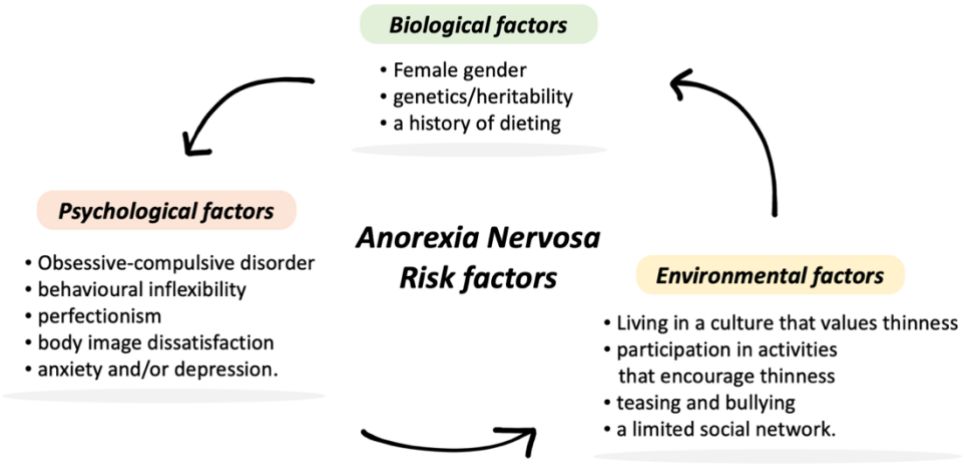

Figure 2 *Behavioral, biological, genetic, psychological, and environmental or cultural influences as risk factors for developing AN. Image arranged from* (Peterson & Fuller, 2019)

Patients with AN, in concomitance with the above-mentioned classical symptoms of reduced energy intake and low BW, display a broad variety of physical and somatic complications that affect several organ systems (Fig 2). All these complications highly depend on the stage of the illness. In the acute phase of the pathology many peripheral organs are affected, and they partially remain damaged even after a full BW recovery. In the acute state, patients with anorexia nervosa present many common complaints such as dizziness, fatigue, or even syncope. In patients with a chronic course, almost every organ system can be affected because of malnutrition and excessive energy loss. They usually display gastrointestinal complications such as decreased intestinal and gastric mobility, constipation, gastric dilation and rupture or esophageal tears; cardiovascular problems such as arrhythmias and cardiomyopathy resulted by a weaken heart muscle, also renal hitches due to the low content of fluids and, in few cases, to the improper use of diuretics and laxatives to lose more weight (Fig 3). More in general, AN patients show dental cavities damages that are secondary to self-induced vomiting, osteoporosis that derive from estrogenic deficiency, anemia, and infertility (Zipfel et al., 2015). Patients with AN have an increased life-time prevalence of autoimmune disease, most prominently type 1 diabetes.

Organ systems or organ	Pathological findings	Leading systems
CNS	Morphological and functional cerebral changes; volume reduction in cerebral grey and white matter	Cognitive deficits
Dental system and parotis glands	Impaired dental status, dental caries, increased serum amylase	Dental caries, enlargement of the parotid glands
Endocrine system and reproductive function	Hypothalamus-pituritary-gonadal-axis, low T_3 syndrome, hypercortisol	Amenorrhoea in women, symptoms of hypothyroidism , depression, elevated stress levels
Cardiovascular system	Hypotension, bradycardia, arrhythmia	Syncope
Gastrointestinal tract	Impaired gastric emptying, gastric dilation, gastro-duodenal ulcers	Constipation, ileus, upper gastrointestinal bleeding
Haematological and immune system	Bone marrow hypoplasia, anaemia with reduced leucocytes and immunoglobulin	Anaemia, (bacterial) infections, compromised immune competence
Renal tract	Hypokalaemia, hypophosphataemia, hypernatraemia	Nephrolithiasis, oedema, syncope
Bone	Reduced bone density (osteopenia) or osteoporosis	Bone fractures and concomitant pain, spinal compression

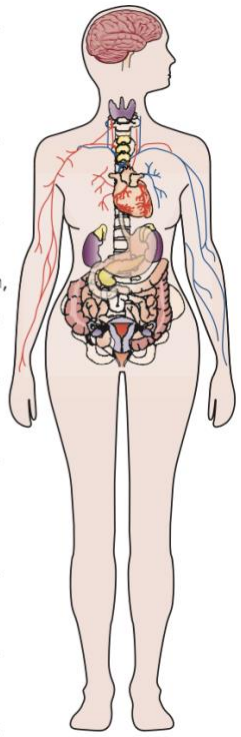

Figure 3 *Impaired organ function in anorexia nervosa* (Zipfel et al., 2015)

The physical activity per se is not considered by the APA as a diagnostic criteria for AN in the DSM-V, however abnormal high levels of physical activity have been observed from the earliest clinical description of AN (Gull, 1997). Nowadays, in clinic the hyperactive behavior is often defined as *problematic use of physical activity* or PPA, and it has been considered to affect 31% to 80% of AN patients (Hebebrand et al., 2003). As a matter of fact, indeed, patients affected by AN use, or in most of the cases *abuse*, physical activity to reach their weight-loss-desire, and the excessive activity is thus for them a strategy to accelerate the process of weight loss, which is already present because of the severe caloric restriction. PPA has been associated with a longer length of hospital stay (Solenberger, 2001) and poor treatment outcome (Taranis & Meyer, 2011). Moreover, protractive PPA behaviors interfere with refeeding strategies and body weight stabilization (Ng et al., 2013) worsening the risk of relapse and chronicity (Strober et al., 1997). Unfortunately, no clear definition, conceptualization, or treatment of the problematic use of PPA in ED patients exist (Rizk et al., 2020), but in light of the strong impact that PPA has in aggravating the course of the disease, a correct analysis of the quality of the exercise, along with a personalized evaluation of the reasons for compulsiveness and *addiction* to exercise would allow more individualized and efficient medical therapies.

In terms of medical assessment, all patients with AN, or only suspected as anorexic, need an initial thorough baseline medical evaluation, which includes an in-depth interview, and a physical examination to evaluate patient's height, post-void undressed weight, BMI, BMI percentile and orthostatic vital signs (blood pressure and pulse rates), along with temperature and respiratory rates. A full physical evaluation should include all organ systems, looking for signs of calluses or parotid enlargement from purging to rule out other causes for malnutrition. Laboratory screening should measure blood count and include a comprehensive metabolic panel to assess for dehydration, hypokalemia, hyponatremia, hypophosphatemia, and transaminitis, which may be found with malnutrition. In addition, markers of nutritional status such as albumin, prealbumin, and transferrin can also be useful to check as well (Weiselberg & Fisher, 2014). Table 2 summarizes all the recommended physical investigations to be done at assessment (Tab 2).

	Essential	Optional
Medical and psychiatric history	Full examination	Structured diagnostic interview (eg, SCID I and II, EDE)
Physical assessment	BMI, heart rate, blood pressure, temperature	In case of severe oedema: albumin, total protein, protein electrophoresis In patients with a long-term course: bone density (DEXA) scan In case of seizures or differential diagnosis: EEG and neuroimaging (CT, MRI) In case of unclear thoracic pain or abdominal symptoms: chest radiograph, abdominal ultrasound, gastroscopy
Blood profile	Full blood count, blood sedimentation rate	In severe anaemia: reticulocytes, iron, ferritin, transferring, vitamin B12
Biochemical profile	Sodium, potassium, calcium, magnesium, phosphate, creatinine, urea, liver enzyme profile, blood glucose	In case of elevated creatinine: creatinine clearance In case of low BMI (<12kg/m²) and in the early phase of nutritional rehabilitation: monitoring of sodium, potassium, phosphate (at least weekly)
Cardiovascular assessment	ECG	In case of cardiovascular abnormalities: Holter ECG, echocardiography

SCID I and II=structured clinical interview for DSM axis I and II disorders. EDE=eating disorder examination interview. BMI=body-mass index. DEXA=dual-energy X-ray absorptiometry. ECG=electrocardiograph.

Table 2 *Initial assessment recommended for the evaluation of patients with AN, or at risk of developing AN* (Zipfel et al., 2015).

In accordance with the practice guidelines released by the National Institute for Health and Care Excellence (National Collaborating Centre for Mental, 2004) and by the American Psychiatric Association (DSM-V, 2013) most of the individuals with AN can be treated as outpatients, but that day-patient and inpatient services are still needed and highly recommended for those with more severe illness and those who do not improve with outpatient care (Zipfel et al., 2015). To date, the fundamental starting point to treat patients affected by AN is the psychotherapy, which includes individual, family, and group therapies. Whenever possible, it is important to involve the family and partners in the assessment and subsequent treatment; in fact, family therapy is one of the most promising approaches at the moment (Couturier, Kimber, and Szatmari 2013; Lock et al. 2010). It aims to reshape the altered perception of oneself by working on the patient's self-esteem, bringing it back to a conception as healthy and physiological as possible, in concomitance with the restoration of complete physical integrity (Carter et al., 2011). Of note, it is worth mentioning that adolescents and adults affected by AN due to a different developmental stage of growth and severity of symptomatology need diverse and specialized therapies (Tab 3), indeed as reported by Zipfel and colleagues, the same psychotherapy might be more or less efficient whether the patient is adolescent or adult (Zipfel et al., 2015).

	Evidence	Effect (evidence level)
Adolescent anorexia nervosa[84]		
Family-based treatment (FBT)	Strong*	+++ (1)
Maudsley family therapy (MFT)	Strong*	+++ (1)
Family system therapy (FST)	Moderate*	++ (2)
Adolescent focused therapy (AFT)	Moderate*	++ (2)
Cognitive behavioural treatment (broad; CBT-b)	Weak/moderate	–/+ (4)
Cognitive behavioural treatment (enhanced; CBT-E)	Moderate*	+ (4)
Adult anorexia nervosa[4,73,83]		
Cognitive behavioural therapy (CBT)	Weak	+
Cognitive behavioural therapy (enhanced; CBT-E)	Moderate*	++
Behavioural therapies (BT)	Weak	–/+
Interpersonal psychotherapy (IPT)	Weak	+
Psychodynamic therapy (PT)	Weak	+
Cognitive analytic therapy (CAT)	Weak	+
Focal psychodynamic psychotherapy	Moderate*	++
Maudsley model of anorexia nervosa treatment for adults (MANTRA)	Moderate*	++
Specialist supportive clinical management (SSCM)	Moderate*	+ (+)

Evidence grades are weak, moderate, or strong. Effect grades are: – for no beneficial effect; -/+ for mixed result or still inconsistent result; + for slight beneficial effect; +(+) for moderate beneficial effect; ++ for moderate and lasting beneficial effect (further improvement shown in follow-up); and +++ for strong beneficial effect (superiority demonstrated in primary outcome of randomised trial). Evidence levels are: 1 for well established, 2 for probably efficious, and 4 for experimental. *At least one multicentre randomised trial or more than one randomised trial.

Table 3 *Behavioral treatments in adolescent and adult patients with anorexia nervosa* (Zipfel et al., 2015).

Psychotherapy is not effective alone, indeed it is often associated with drug therapy. Antidepressants, including selective serotonin reuptake inhibitors (SSRI) have been observed to improve patients' depressive condition, but have little effect on relapse prevention (Attia et al. 1998). From the literature, it emerges that scientists and clinicians believe that antidepressants neither improve weight gain nor reduce eating disorder or other psychological symptoms in the refeeding phase (Aigner et al., 2011). Thus, the utility of antidepressants for relapse prevention in the post-weight restoration phase remains uncertain. Anxiolytics such as benzodiazepines did not have any efficacy in the treatment of AN, they resulted just as symptomatic medications for anxiety, that in many cases is comorbid to AN (Kaye et al., 2004). Cannabinoid receptor agonist, ghrelin receptor agonists and tumor necrosis factor are currently under investigation for their putative role in treating AN, because of their ability to promote appetite (Tortorella et al., 2014), but still no one of these drugs have proven efficacy. In

summary, all the currently available pharmacological interventions proposed to treat AN have no efficacy in curing the disease. The main reason behind the high rate of treatment failure is the fact that these treatments are only symptomatic and do not target the cause of the disease.

Therefore, the urgent need of discovering the still unknown biomarkers to target, further point to the importance of the preclinical research, and the use of preclinical models in filling the gaps of clinics. In fact, preclinical models allow to investigate at cellular and molecular levels the alterations induced by the pathology, with the final goal to translate these findings for human treatments.

1.3 *Reward circuit unbalance: what feeds the vicious loop?*

The ability to maintain self-induced starvation behaviors with hyperactive regimens of activity, despite the severe condition of malnutrition and emaciation, remains one of the biggest open questions concerning the pathophysiology of AN. To deeply understand this disturbance, it is necessary to understand the neurobiological mechanisms that allow or promote the persistent choice of inadequate caloric intake together with the increase of physical activity despite the abnormal reduction of energies (Steinglass & Walsh, 2016). These disturbances hide a diminished capacity for AN patients to experience pleasure or reward for natural stimuli, such as food, thus they do not suffer the limitation of food, on the contrary they are satisfied without eating. This inability to appreciate food emerges to be due to a hypo-responsiveness of the reward system (Kaye et al., 2009; Wagner et al., 2007; Zink & Weinberger, 2010). Furthermore, evidence from Fladung and colleagues demonstrate an increased ventral-striatal activity in AN patients in response to pictures of underweight women, and these stimuli that are closely linked to starvation seem to have rewarding properties for these patients (Fladung et al., 2010). Thus, these findings provide preliminary evidence of the positive motivational valence of abnormal stimuli, recognized as *disorder-specific stimuli*, that in the case of AN are rewarding and that might drive for self-starvation.

Over time several neural systems have been considered in the etiology of AN, including the cognitive self-regulatory control, motivation and reward processing, and hunger regulation systems (Campbell et al., 2011; Friederich et al., 2010; Hasan & Hasan, 2011; Wierenga et al., 2014). In particular, in the literature, four comprehensive etiological models of AN have been proposed. In the oldest one, AN was viewed as a complete state of anhedonia in which a reduced capacity to experience rewards is linked to a hyporesponsive DA reward system (Davis & Woodside, 2002; Kaye et al., 2005). Then, the second model proposed rely on the alteration of the reward circuit based on the overcontrol of dorsal brain structures over ventral limbic structures, with the effect of reduced response to the aversive signals related to food experience, and shifts behavior towards cognitive goals associated with food avoidance and weight loss (Brooks et al., 2011; Kaye et al., 2009). The third model was based on the concept of "reward contamination", in which inappropriate overlapping of neural pathways develop as a result of paradoxically rewarding behaviors, such as food restriction that overlap with circuits of punishment (Keating, 2010; Keating et al., 2012). Thus, AN develops because of the rewarding effects of self-starvation and persists even though prolonging such behavior results in a

punishing state (Scheurink et al., 2010; Zink & Weinberger, 2010). The most recent etiological model of AN has been proposed by Walsh and colleagues, and emphasize the importance of learning and the formation of habits over goal-oriented behaviors in the reiteration of maladaptive behaviors (Walsh, 2013).

For years the second abovementioned model of AN, has been the most accredited etiological model of AN that allowed to explain the food intake refusal involve an imbalance between central reward and inhibition mechanisms, in which hedonic drivers of food intake are slowed down and the inhibitory control powered. This model comprises *top-down* versus *bottom-up* elements, in which top-down modulatory mechanisms reflect prefrontal cognitive control regulated by the medial prefrontal cortex (mPFC), while bottom-up limbic and interoceptive processes, mainly mediated by the Nucleus Accumbens (NAc) reflect the integration of input from systems that regulate factors such as homeostatic need, responses to reward, and motivational drive (O'Hara et al., 2015). In other words, two main neural networks interact to encode AN behaviors: the limbic and the cognitive circuits. The first involves the amygdala, the ventral striatum and the NAc, that together with the insula and the anterior cingulate coordinate the affective response to different pleasurable stimuli including food and feeding. These limbic neurocircuits are formed by dopaminergic projections that originate in the ventral tegmental area (VTA) and project to the NAc, where are placed inhibitory GABA (gamma-aminobutyric acid) receptors, that cooperate in balancing rewarding responses. The cognitive counterpart, instead, ponders, modulates and regulates the predisposition to undertake the affective behavior, and the regions responsible for this fine tuning on the more impulsive limbic system are more dorsally positioned, such as the dorsal prefrontal cortex and parietal regions (Foldi et al., 2017a). Of note, glutamatergic projections connect the two circuits as a *file rouge* between limbic and cognitive networks, originating from the frontal cortex extending between the orbito-frontal cortex, anterior cingulate cortex and ventromedial prefrontal cortex and projecting to mesolimbic pathways, thus influencing the hedonic perception of food and other rewards (Fig 4) (Bimpisidis & Wallen-Mackenzie, 2019; Der-Avakian & Markou, 2012; Gleich et al., 2015).

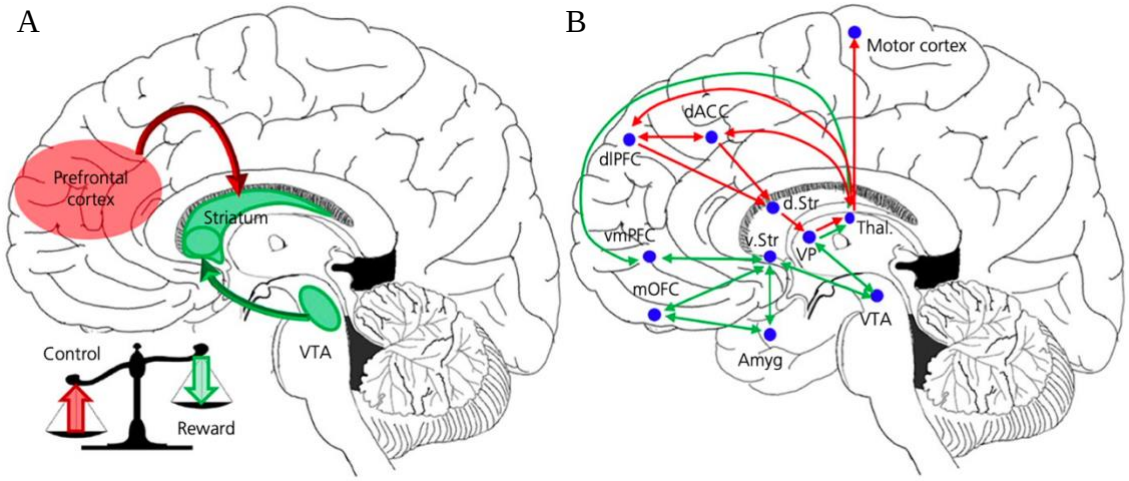

Figure 4 *Neurocircuitry modulating reward and inhibition responses in AN* (Foldi et al., 2017a).
Panel (A) show the imbalance between the overactive prefrontal regions (red), responsible for judgment and decisions, and the underactive mesolimbic regions (green), responsible for emotions and reward. Panel (B) illustrate the different neural pathways that contribute to executive control circuits (red arrows) and reward circuits (green arrows) have been derived from human studies.

Abbreviations: VTA, ventral tegmental area; Amyg, amygdala; VP, ventral pallidum; Thal., thalamus; v.Str, ventral striatum; d.Str, dorsal striatum; mOFC, medial orbitofrontal cortex; vmPFC, ventromedial prefrontal cortex; dlPFC, dorsolateral prefrontal cortex; dACC, dorsal anterior cingulate cortex

Results obtained from functional neuroimaging studies in AN patients support this hypothesis demonstrating that the hyperactivation of top-down cognitive control networks exerts control over bottom-up appetitive responses. In brief, they found that exaggerated neural activity in the dorsolateral prefrontal cortex (Ehrlich et al., 2015) leads to heightened cognitive control, and reduced neural activity in the striatum (Frank et al., 2016), resulting in depressed reward function. Thus, the boosting of top-down control over the depression of bottom-up responses emerge for this patients as the pathological desire to be thin, also explaining their typical overcontrol behavior (Kaye et al., 2009).

Despite the etiopathogenesis of AN is still unknown, the current proposed view of AN etiology may be considered as a balanced compromise among the four models proposed over time (Fig 5). A view that is consistent with other theoretical frameworks in the field, in which AN does not appear to be a generalized inability to experience reward, but rather as a multifactorial disorder originated by the combination of neurobiological and psychophysiological factors, in which the altered reward processing, the *top-down* over the *bottom-up* control, compulsivity

and habits formation, and the exaggerated cognitive control play a major role in the nature of the anorectic symptomatology (O'Hara et al., 2015).

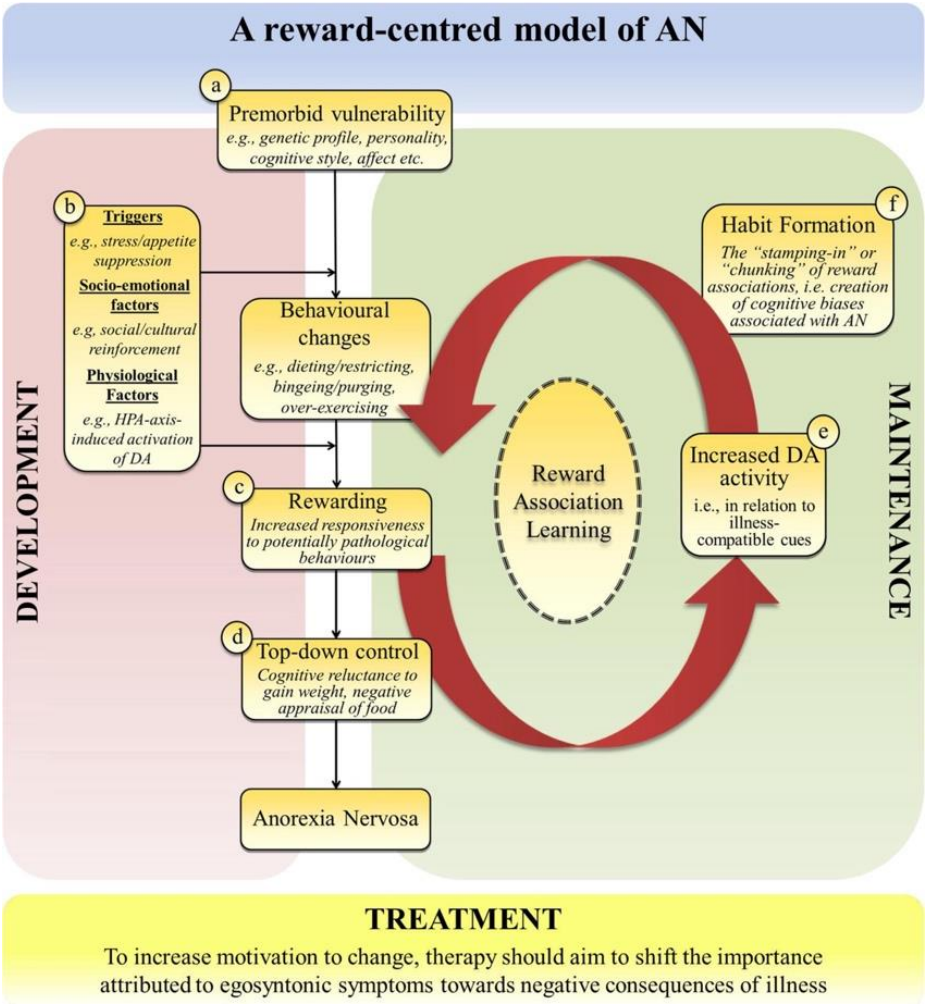

Figure 5 *A reward-centered model for the development and maintenance of anorexia nervosa (AN)* (O'Hara et al., 2015)

In the context of reward circuit unbalance and dysregulation of central processes of reward and inhibition, it is worth mentioning the emerging implication of peripheral hormones and in particular of appetite modulators such as ghrelin and leptin. It has been proposed that both their "homeostatic" and "non-homeostatic" functions may have a role in the pathophysiology of EDs (Monteleone & Maj, 2013). Leptin is an adipocyte-derived factor that behaves as a hunger suppressant signal by informing the central nervous system on the amount of energy stored in the body adipose tissue (Blundell et al., 2001). On the other hand, ghrelin is primarily produced by endocrine cells in the stomach and behaves as a hunger hormone stimulating appetite and

promoting gastric emptying (Cummings et al., 2001). Of note, both leptin and ghrelin exert a non-homeostatic regulation on hedonic and motivational components of reward. In. fact, it has been found a correlation between the modulatory effect of leptin on reward-related behaviors in the development of overexercise in AN patients. Moreover, Verhagen et al. (2011) demonstrated that leptin injection into the VTA suppresses running wheel activity in rodents exposed to the activity-based anorexia (ABA) rat model, suggesting that the ABA-induced reduction of leptin signaling in the VTA via increased mesolimbic dopaminergic activity leads to an enhancement of rewarding properties of running wheel activity (Verhagen et al., 2011). Akin leptin, ghrelin exerts a non-homeostatic function as well, it has been demonstrated that ghrelin modulates the locomotor activity of animals enhancing explorative behaviors, which in turn may have a potential readout in facilitating food-seeking and hunger-related survival behavior (Jerlhag et al., 2007).

Although the exact implication of peripheral hormones in regulating reward and, thus in affecting eating behaviors in EDs such as AN, is still under debate, there are promising evidence suggesting that appetite modulators might play a crucial role in the central processes of food-related stimuli contributing to the long-lasting maintenance of aberrant eating behaviors.

1.4 *The neurobiology behind: from onset to maintenance*

Evidence exists, in both AN patients and animal models, that maladaptive reorganization of the mesocorticolimbic structures may represent the neurobiological underpinning of the motivational mechanisms underlying the anorexic phenotype (Foldi et al., 2017b; Frank et al., 2018; Ho et al., 2016). In this scenario, dopamine (DA) has been widely described as the focal neurotransmitter of the mesocorticolimbic reward system involved in several processes that mediates hedonic responses, particularly for its role in behaviors associated with motivation and compulsiveness, such as drug addiction (Koob & Volkow, 2010). Dopaminergic neurons of the midbrain innervate areas that include the NAc, PFC, hippocampus, and amygdala (Haber & Knutson, 2010; Kalivas & Volkow, 2005). DA is of relevance in the clinical profile of AN because it is involved in regulating motor activity, feeding, and reward-motivated behavior (Wise, 2008). However, from the literature emerge that DA is not sufficient to mediate changes in the general hedonic response to salient stimuli, or in the new-learning and habits formation, that are indeed central behaviors in the onset and maintenance of AN.

In this context, there are evidence highlighting the contribution of other neurotransmitters in the modulation of the limbic system, specifically in the regulation of anxiety behaviors in animal models of AN, such as the GABAergic system (Aoki et al., 2017). Of note, it has been demonstrated that the GABAergic innervation of pyramidal cells, as well as the presence or absence of α4βδ-$GABA_A$ receptors at spines of CA1 pyramidal cells are tiny associated with resilience and vulnerability to the development of AN in rodents (Aoki et al., 2014). In this regard, it has been further investigated the presence of α4βδ-$GABA_A$ receptors at spines and, recently, it has been demonstrated that its accumulation at the synaptic cleft of excitatory synapses, more than at non-synaptic portions of the plasma membrane, strongly contributes to the suppression of the food restriction-induced wheel running and to lowering the weight loss in anorexic rodents (Aoki et al., 2018).

Alongside the involvement of the dopaminergic innervation in modulating the salience of food reward, physical activity, and vulnerability to AN (Beeler et al., 2020; O'Hara et al., 2015), and with the contribution of the GABAergic projections involved in resilience to AN (Aoki et al., 2017), the integration of glutamatergic excitatory inputs to DA neurons is crucial in regulating and engaging dopaminergic signals during habit learning (Wang et al., 2011).

Glutamate is the primary excitatory neurotransmitter of the central nervous system (CNS) and is a key neuromodulator of synaptic plasticity and neural circuit formation. In fact, despite the knowledge about the role of the glutamatergic system in AN is still poorly understood, many neuropsychiatric disorders, such as schizophrenia (Itokawa et al., 2003) and addictive disorders (Caffino et al., 2021; Piva et al., 2021; Smaga et al., 2021), are characterized by the disruption of glutamatergic synapse function, that cause excitation/inhibition imbalances (O'Donovan et al., 2017; Volk et al., 2015). Of note, a study of magnetic resonance spectroscopy (MRS) conducted by Godlewska and collegues with high field 7Tesla-MRS to measure metabolites content, recently showed that female AN patients are characterized by significant reduced concentrations of glutamate widespread in the brain (Godlewska et al., 2017), highlighting the interest in understanding in depth the potential role of glutamate in the pathophysiology of AN.

Glutamate interacts with various membrane receptors, and two groups of glutamatergic receptors have been described based on their mechanism of action: 1) ionotropic glutamate receptors (iGluRs) form ion channels that activate a postsynaptic current when the neurotransmitter binds to the receptor; 2) metabotropic glutamate receptors (mGluRs), on the contrary, are G-protein coupled receptors (GPCRs) that indirectly activate ion channels located on the plasma membrane through a signal cascade involving G proteins. Ionotropic receptors are faster in transmitting information while the activation of metabotropic receptors is associated with a more prolonged stimulus.

iGluRs form cation-permeable ion channel receptors and can be subdivided into AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) receptors, kainate receptors and NMDA (N-metil-D-aspartate) receptors (Traynelis et al., 2010). AMPARs mostly consist of two GluA1and two GluA2 subunits. NMDARs mostly consist of two obligatory GluN1 and two accessories GluN2A-2B subunits, in which GluN2A-2B directly bind to PSD-93/PSD-95/SAP102 scaffolding proteins (Fig 6).

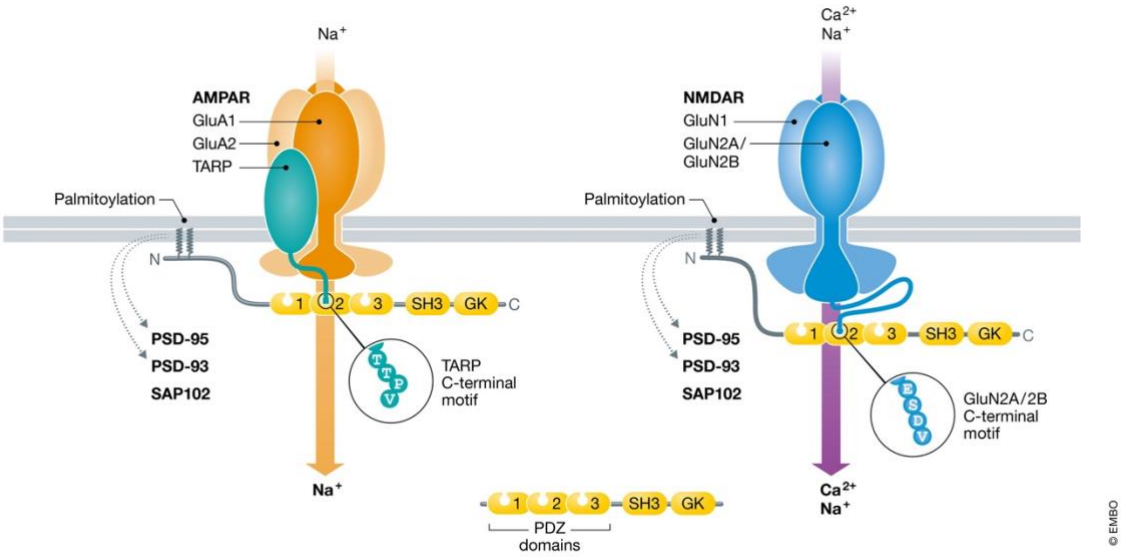

Figure 6 *Structural Organization of post synaptic AMPA and NMDA receptors.* (Patriarchi et al., 2018) AMPARs are mainly Na^+- and K^+-permeable, whereas NMDARs also conduct Ca^{2+}. Postsynaptic anchoring of PSD-95 and some PSD-93 isoforms but not of SAP102 requires palmitoylation of their N-termini (depicted in zigzag), which fosters their interactions with AMPARs and NMDARs.

The excitatory glutamatergic synapse is recognized as a tripartite structure in which the main components are the pre-synaptic terminal, the post-synaptic terminal and the glial cell. At the presynaptic terminal, action potentials trigger the fast release of synaptic vesicles containing neurotransmitters. Synaptic vesicles are docked at the active zone and primed for exocytosis by protein complexes. The release of glutamate is closely aligned with the postsynaptic AMPA and NMDA receptors that are stably anchored in the postsynaptic density (PSD), a complex molecular machine containing a plethora of scaffolding proteins and signaling molecules, located in the post-synaptic terminal (Fig 7) (Scheefhals & MacGillavry, 2018). In summary, iGluRs, that are enriched in the PSD, are essential mediators of the response to stimuli and synaptic adaptations of connections in a highly plastic manner. AMPA receptors (AMPAR) have a lower affinity for glutamate than NMDA receptors (NMDAR). Conversely from NMDAR, AMPAR are weakly permeable to external calcium ions (Jonas et al., 1994), and their mechanism of action is required for an initial excitatory potential when the neurotransmitter is present in the synapse. In presence of glutamate, AMPAR are activated, and the membrane depolarizes, favoring the essential removal of Mg^{2+} ions from NMDA channels, thus promoting their activation. When NMDAR are activated the influx of different cations is allowed, especially of Ca^{2+}, which is the most relevant ion implicated in CNS physiology and pathology. The NMDAR exhibits a complex gating mechanism, requiring not

only glutamate binding, but also cofactors binding and cellular depolarization. (Marmiroli & Cavaletti, 2012). The Calcium Ca^{2+} influx through NMDAR ion channels activates intracellular kinases and phosphatases, thereby altering the characteristics of the synapse and giving the basis for neuronal transmission.

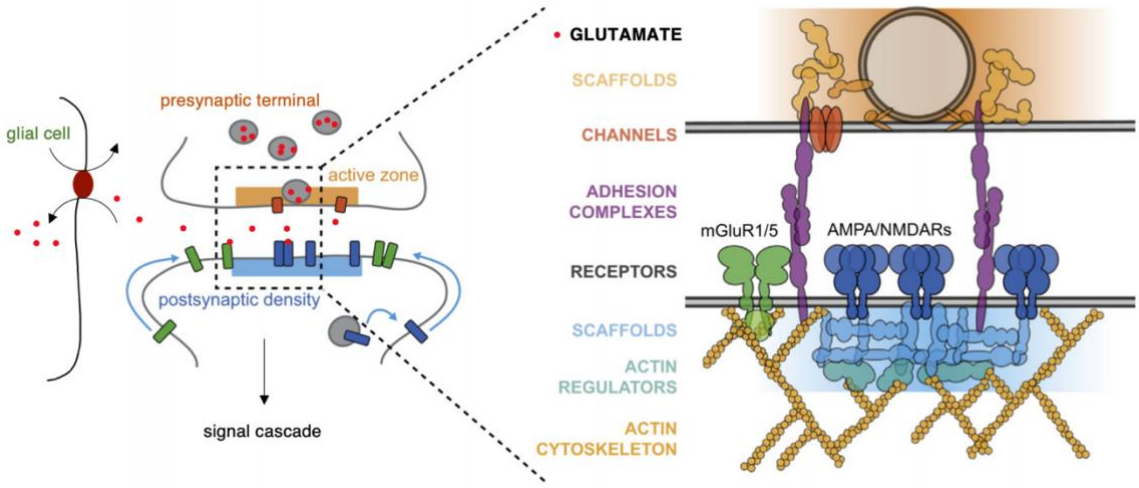

Figure 7 *Schematic representation of the organization of the tripartite glutamatergic excitatory synapse*. *Adapted from* (Scheefhals & MacGillavry, 2018)

It has been shown that NMDA and AMPA receptors plasticity is crucial in the onset of memory deficits (Franchini et al., 2020; Henley & Wilkinson, 2016). For instance, GluN2A-knockout or GluN2A C-terminus-deficient mice exhibit impaired spatial working memory (Bannerman et al., 2008), and even more interestingly, GluN2A-containing NMDARs have been implicated in several pathological conditions, such as depression (Boyce-Rustay & Holmes, 2006) and anxiety (Sun et al., 2013). The prolonged decrease of GluA2-containing AMPARs in the membrane surface of vulnerable neurons it has been demonstrated to mediate a switch from calcium impermeable (CI) -AMPARs to calcium permeable (CP)-AMPARs which has been found as a causal factor in neuronal death (Hanley, 2014). Of note, in the activity-based anorexia (ABA) experimental model of AN in rodents it has been found increased levels of postsynaptic GluN2B-subunits of NMDA receptors in the dorsal Hip of anorexic rodents, especially among the animals showing the most severe weight loss (Chen et al., 2017). Furthermore, Bilash and colleagues in their recent work published in 2021 demonstrate the important role of the glutamate transporter-1 (GLT-1) in the Hip of rodents developing AN. In particular, they demonstrate that animals expressing higher levels of GLT-1 at excitatory

synapses are able to minimize the severity of ABA, indeed high levels of GLT-1 negatively correlates with weight loss and compulsive exercise (Bilash et al., 2021).

Thus, glutamatergic receptors are key players in neuroplasticity, and are able to convert specific patterns of neuronal activity into long-term changes in synapse structure and function that are thought to underlie higher cognitive functions (Paoletti et al., 2013), and might be responsible of the maladaptive plasticity that underlie psychiatric disorders, such as AN. Nevertheless, several distinct aspects about the involvement of glutamatergic receptors and excitatory structural markers still need to be elucidated to fully understand how the glutamatergic system is involved in the different phases of AN and can be effectively targeted to treat this psychopathology.

1.5 *The neurometabolic axis: a periphery-to-brain crosstalk*

In humans and rodents, it is known that physical activity and in particular balanced endurance exercise promote beneficial effects on brain health and cognitive function. Also, it has been demonstrated that physical activity ameliorates certain neurological diseases, such as depression, epilepsy, stroke, Alzheimer's, favoring better outcomes and improving the quality of life of patients. In particular, it has been reported that exercise increases the size and the blood flow in human hippocampus, a brain region involved in learning and memory, and induces morphological changes in dendrites and dendritic spines, promoting synaptic plasticity and, of note, *de novo* neurogenesis in the dentate gyrus in various mouse models of exercise (Cotman et al., 2007; Mattson, 2012). One important molecular mediator for these beneficial responses to exercise is the neurotrophin BDNF, the well-known *brain-derived neurotrophic factor* (Jodeiri Farshbaf et al., 2016; Vaynman et al., 2004).

BDNF is the most abundant neurotrophin in the brain, responsible for many vital processes such as neuronal growth and neuroplasticity, but it also plays key roles in the homeostatic regulation of food intake and energy expenditure, stress responsivity, and reward processing (Barde, 1989; Ghitza et al., 2010; Gomez-Pinilla et al., 2002; Lebrun et al., 2006; Wang et al., 2010).

BDNF binds to the high-affinity tropomyosin-related kinase receptor B (TrkB) leading to receptor activation through dimerization and autophosphorylation. The activated TrkB receptor recruits several downstream intracellular phosphorylation cascades (Fig 8) (Benarroch, 2015). Among all the neurobiological effects of BDNF-TrkB receptor signaling pathway, recently it has been demonstrated that the BDNF-TrkB pathway play a crucial role in activity-dependent strengthening of excitatory synapses in different brain regions (Kellner et al., 2014). In addition, BDNF application to cortical and hippocampal neurons positively modulates structural plasticity in vitro, promoting dendritic spine remodeling (Mcallister et al., 1995, 1996; Horch et al., 1999). Interestingly, the presence of postsynaptic BDNF in dendrites and spines of hippocampal CA1 pyramidal neurons were observed and TrkB activity was monitored in single dendritic spines of CA1 pyramidal neurons in cultured murine hippocampal slices. Harward and collegues also show rapid, glutamate-uncaging-evoked, time-locked BDNF release from single dendritic spine, demonstrating that the post-synaptic BDNF-TrkB

signaling pathway activity is crucial for structural and functional plasticity at a single dendritic spine level (Harward et al., 2016).

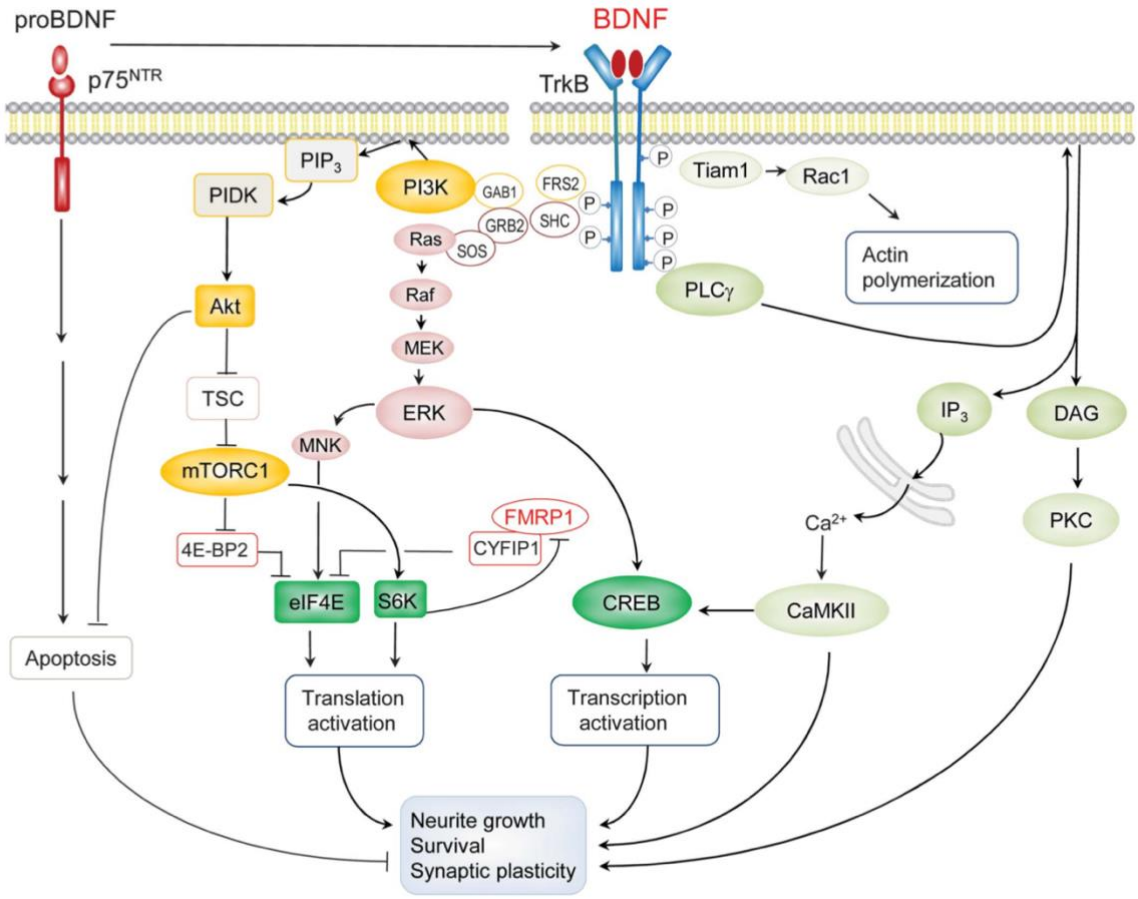

Figure 8 BDNF-mediated intracellular signaling pathway (Benarroch, 2015)

Therefore, in a pathological condition such as AN, in which physical exercise is extreme and turns to obsession, the energy expenditure is excessive, and the reward processes are likely to be compromised, BDNF has been hypothesized to be an important molecule in the development and maintenance of AN (Baker et al., 2017; Bulik et al., 2007; Ho et al., 2016). At clinical level, circulating concentration of BDNF was reduced in patients affected by AN (Brandys et al., 2011), an effect that has been normalized, or even slightly increased, with weight-recovery (Ehrlich et al., 2009). Moreover, a family-based association study conducted on European populations gives additional support to the association between the polymorphism of *Bdnf* gene and the restrictive type of AN, strengthening the correlation between the Val66Met allele and increased vulnerability towards AN (Ribases et al., 2005). In line with the clinical evidence, results from studies on mice models confirmed the involvement of BDNF in the pathophysiology of EDs (Kernie et al., 2000; Rios et al., 2001).

In 1995, it has been demonstrated in rodents that central infusion of BDNF leads to severe appetite suppression and weight loss in rats (Pelleymounter et al., 1995). More recently, in 2018, Ho and colleagues show that the induction of the ABA model in mice alters the expression of BDNF transcripts in different areas of the mesocorticolimbic circuit (Ho et al., 2016), highlighting the contribution of wheel running-induced increases of hippocampal BDNF as a critical factor in the induction of anorexic behaviors.

Moreover, BDNF has been proposed as the central neural mediator of the beneficial effects of physical exercise (Wrann et al., 2013), while PGC1α (peroxisome proliferator-activated receptor-γ co-activator 1α) has been considered as the peripheral mediator of the effects induced by exercise (Finck & Kelly, 2006). In 2012, Bostrom and colleagues identified a PGC1α-dependent myokine, FNDC5, as a transmembrane fibronectin type III domain-containing protein 5 that after proteolytic cleavage is released in blood circulation acting as a hormone-like peptide. PGC1α is involved in a wide-range of metabolic processes, it mediates the programming of energy metabolism in transcriptional biological systems; it controls mitochondrial biogenesis, angiogenesis, fiber-type switching, and oxidative metabolism in many cell types, and it controls FNDC5 gene transcription. The authors discovered that FNDC5, induced following exercise, is then proteolytically cleaved from the cellular membrane of myocytes where it is located and released in the blood circulation as a small peptide (112 amino acids), named as Irisin (Bostrom et al., 2012) (Fig 9).

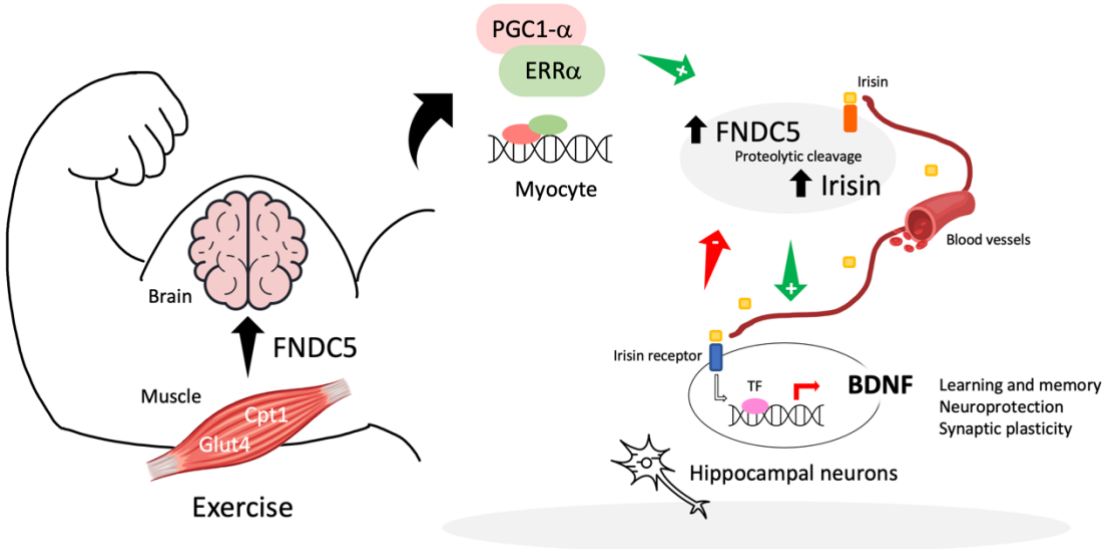

Figure 9 *Schematic representation of the periphery-to-brain axis PGC1α/FNDC5/Irisin/BDNF*

Human clinical studies have confirmed a positive correlation between the level of exercise performance with increased FNDC5 expression and circulating irisin (de Oliveira Bristot et al., 2019; Huh et al., 2012; Lecker et al., 2012). Moreover, recent findings demonstrated that FNDC5/Irisin stimulate the expression of BDNF in the hippocampus (Wrann et al., 2013), establishing an interesting close link among physical exercise, peripheral PGC1α/FNDC5, circulating irisin and BDNF at central level, specifically in the hippocampus, a key brain areas for long-term memory processes and learning.

Despite the role of BDNF as a molecular mediator of neuroplasticity at central level, emerging findings report that BDNF play widespread roles in regulating energy homeostasis by modulating glucose metabolism and molecular patterns of feeding and physical activity in peripheral tissues (Marosi & Mattson, 2014). Accordingly, recent data demonstrate that the expression of BDNF and TrkB receptor is present not only in nervous cells but also in skeletal muscle, liver, and adipose cells (Marosi & Mattson, 2014). In particular, it has been demonstrated that BDNF influences the functionality of multiple cell types involved in glucose metabolism in the body including pancreatic β-cells, by inducing insulin production, hepatocytes, causing decreased glucose production, and skeletal muscle by increasing insulin sensitivity (Matthews et al., 2009; Yamanaka et al., 2007). Of note, in both humans and rodents it has been demonstrated that inherited BDNF deficiency cause severe obesity, and selective BDNF knock-out in mice cause hyperglycemia and diabetes (Gray et al., 2006; Kernie et al., 2000; Teillon et al., 2010), further pointing to BDNF not only as a key player in regulating neuroplastic processes in neurons, but also as a critical regulator of cell metabolism and energy balance in peripheral cell types.

1.6 *The Activity-Based Anorexia experimental model*

The absence of effective pharmacological treatments for AN, together with limited prevention strategies, is one of the major problems deriving from the lack of knowledge about the molecular markers underlying the pathology. To address this need, neuroscientists develop an animal model to study how neural circuits may contribute toward vulnerability to AN. One of the major difficult challenges in studying psychiatric disorders from a preclinical point of view, such as depression or schizophrenia, is the identification of the most appropriate animal model able to reproduce the core features of the disease. The use of an animal model of AN enables the systematic study of biological factors involved in the development and maintenance of self-starvation in a controlled environmental setting.

Over the years, different animal models of AN has been proposed and classified in "environmental models" or "genetic models" (Scharner & Stengel, 2020). Briefly, the environmental model classification includes rearrangements of the environmental settings properly set up by the experimenter to induce body weight loss without any directed manipulation on the animal. For example, the ABA, the Dehydration-induced model (DI), the Valine-deficient diet (VD) or the Separation-induced anorexia (SI) are models in which by modifying environmental conditions, such as the liquid or solid diet (DI, VI), by introducing a wheel (ABA) or inducing stress through isolation (SI), body weight loss is obtained (Carrera et al., 2014; Hao et al., 2001; Nakahara et al., 2012; Reyes-Haro et al., 2015). Conversely, genetic models involve genetic manipulations to induce weight loss, such as the induction of the *anx* mutation and the consequent generation of anx/anx mice (Mercader et al., 2008), or the induction of the overexpression of the Dopamine receptor 2 (D2) that generates D2-Cre BAC Transgenic Mice (Welch et al., 2021).

A fundamental issue in mimic AN in animals is the reproduction of "voluntary" food restriction rather than imposed starvation. As mentioned before, different animal models has been set up, but among them, the most valid and widely used model has been the ABA rat model, proposed for the first time from Routtenberg and Kuznesof in the past 1967 (Routtenberg & Kuznesof, 1967).

As explained by Chowdhury and colleagues, the ABA model is a bio-behavioral phenomenon described in rodents that mimic the key symptoms of anorexia nervosa (Chowdhury et al., 2015). In the table below a comparison between the features of AN disease in humans and the same characteristics reproduced in the ABA animal model is presented (Table 4).

Table 4. *Comparing features of anorexia nervosa in humans and in the activity-based anorexia rat model.*

Anorexia Nervosa		Activity-based Anorexia (ABA)
Severe dietary restriction, self-starvation	⟺	Severe dietary restriction, self-starvation
Marked weight loss	⟺	Marked weight loss
Hyperactivity/excessive exercise	⟺	Hyperactivity/increased wheel running
Amenorrhea in females	⟺	Loss of estrous cycle function in adult females
Increased vulnerability in adolescence	⟺	Increased vulnerability in adolescence
Predominantly females	⟺	Sex differences dependent on age of animal (both male and female rats can develop ABA)
Inability to maintain healthy body weight	⟺	Refusal to maintain body weight by engaging in behaviors that promote weight loss rather than conserving energy and/or binging on food when it is available to maximize resources
Fear of gaining weight or becoming "fat" Body image disturbances	}	Cannot be modeled in the rat

Note. Adapted from "Animal Models of Eating Disorders," by N.M. Avena (ed.). *Neuromethods, 74*. (pp. 283). 2013 Copyright by Springer Science+Business Media, LLC

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Adapted from (Barbarich-Marsteller, 2013).

The outcomes impossible to mimic in an experimental model of a pathological condition are those related to personal psychological implications, particularly related, in AN, to the fear of gaining weight or becoming fat, and all the body image disturbances concerning the body shape. Even though we are aware that no animal model can mimic all aspects of a complex psychiatric disorder such as AN, the ABA model, the gold standard in the field, has the potential to provide useful insights into the mechanisms underlying the maintenance of these maladaptive behaviors. For example, the auto-imposed severe dietary restriction can be reproduced by reducing the feeding schedule of the animals, giving them the possibility to eat during a limited time-window during the day. Moreover, the excessive exercise that AN patients perform during days and nights is reproduced in the animal model by means of a mechanical activity-cage (Fig 10) in which rodents can run without limits. The only limitation of the running activity that, in most of the cases, is imposed is during the restricted feeding schedule, in order to allow the animals to eat, rather than to run, at least during this period, to let the animals to acquire a sufficient amount of energy intake to proceed further in the experiment. As consequence of these two combined conditions, self-induced body weight loss typical of AN is reproduced.

Specifically, when rodents with free access to a running wheel perform voluntary exercise and experience food restriction, they become hyperactive, running more than animals with free access to food. The experimental procedure involves food restriction with food available for 2 h per day, and the possibility to perform physical activity, that is allowed giving to rodents free access to a running wheel (Fig 10) for the rest of the day (22 h per day).

Figure 10 *Activity wheel (*BIO-ACTIVW-M – from *Bioseb, in vivo research instruments).*

Under these conditions animals develop the anorexic phenotype, that is largely represented by the enhancement of exercise which culminate in hyperactivity, reduction of food intake and dramatic weight loss. It is important to mention that in this paradigm the food restriction consists in the limitation of food in terms of time of food availability, and not in term of quantity of food available that rodents can eat. Thus, since the rodents have the possibility to eat the preferred amount of food in a limited amount of time, the observed reduction of food intake in ABA rodents can be defined as "voluntary", and not as "forced" food intake reduction. Food restricted animals prefer exercise over feeding, even during the period of free food access (Carrera et al., 2014), that explain in the first instance the reduction in food intake, thus leading to severe weight loss that may be fatal, unless animals are removed from the experimental setting (Barbarich-Marsteller et al., 2013; Chowdhury et al., 2015; Gutierrez, 2013).

For these reasons, in order to minimize animal distress due to the induction of the phenotype, and the number of animals used, the experimental protocol has to follow specific criteria during the whole experimentation. Criteria used in ABA research to prevent animals' death are: (1) minimal food intake (i.e., less than 1 g); (2) minimal body temperature (i.e., less than 33°C),

and/or (3) a maximum body-weight loss (i.e., 25% of free-feeding weight) (Carrera et al., 2014). In our experiments, two out of three criteria (1 and 3) have been monitored, while the temperature was manually assessed by the operator. To prevent excessive weight loss below 75%, and animal death, during the 2 h of feeding the wheels were blocked to all the animals exposed to the combination of food restriction and free exercise. Even though it has been widely accepted that the ABA model nicely recapitulate human AN, it has several limitations related to the complexity that identify psychiatric disorders. In particular, rats do not experience "weight phobia", nor do they spontaneously restrict food intake, even though their food consumption is lower compared to rats subjected only to the food limitation (Gutierrez, 2013). Moreover, immediately after the beginning of the food-ad libitum refeeding schedule during the period of body weight recovery, the ABA rats will refeed without any problem, which is not exactly what happens in humans.

Aware of these limitations, and aware of the fact that the ABA model does not allow to investigate the primary cause that leads to the aberrant diet and exercise behavior in humans, it is indeed an excellent model of AN since it exhibits the two major physiological manifestations of the disease: self-induced pathological body weight loss, and voluntary excessive exercising. With this model we are allowed to explore AN neurobiology, focusing on non-social, non-cultural risk factors, neuronal circuit changes and neuroplastic mechanisms associated with AN (Aoki et al., 2017), that ultimately can generate useful clinical leads for its treatment.

2 Aim

Anorexia nervosa (AN) is a disabling psychiatric disorder that mainly affects pubescent girls during adolescence, a vulnerable window of development, radically impacting their health, growth, psyche, social life and future. More than 1-2% of young women and 0.3-0.7% of young men of the western countries suffer from AN. Moreover, AN has a mortality rate that exceeds other psychiatric disorders, with the highest rate of relapse and suicide.

Patients suffering from AN are characterized by an intense fear of gaining weight that combined with a disturbed body image concern motivate the loss of weight, exploiting a severe dietary restriction and intense physical exercise, that in turns induce tremendous emaciation. Despite this clear symptomatology, anorexic patients display other puzzling symptoms, such as emotional fluctuations and obsessive and compulsive behaviors. Altogether these symptoms generate an *out-of-control* spiral in which the complete control over the food intake and the body shape became extremely rewarding for the patient unceasingly feeding the vicious loop of AN. The unknown etiology of this disorder makes the current therapies elusive, often costly and time consuming, and not effective to the cause of the disease. Nowadays AN still represents a neglected field for preclinical research, and the lack of knowledge regarding the biological targets and the molecular mechanisms implicated in the onset of the disease or in its long-lasting maintenance further aggravate this clinical burden.

Interestingly, a growing body of evidence supports the hypothesis that long-term alterations in energy intake are associated with dysfunctions in the central nervous system (CNS), such as brain volume changes, altered balance between reward and inhibition mechanisms and, at neurobiological level, alterations in the levels of neuropeptides as ghrelin, leptin, corticotropin-realising factor, and endocannabinoids (Hasan & Hasan, 2011; Monteleone & Maj, 2013). Moreover, AN is not only a brain disorder, but it is rather a systemic disease that impacts the whole body of a patient, affecting circulating hormones, peripheral organs, and metabolic active tissues such as skeletal muscles, suggesting a crosstalk between peripheral signals and brain functions.

In this scenario, our hypothesis is that dysregulation of this periphery-to-brain neurometabolic crosstalk may play a crucial role in the maintenance of the anorexic phenotype. In particular, intense exercise coupled with low caloric intake, two hallmarks of AN, might activate periphery-to-brain signals, via altered neurometabolic pathways in specific areas of the brain and that such dysregulation drives weight loss seeking through food restriction, compulsive

exercise, and cognitive impairments. Thus, the main goal of the present thesis is to investigate the biological mechanisms and cognitive-related dysfunctions involved in the acute phase of AN, and after body weight recovery, during a critical developmental period, i.e. the adolescence, linking molecular alterations in peripheral organs to neuroplastic dysregulations in the brain.

In particular, the major aims of this work are: 1) unraveling the molecular mechanisms of altered metabolic state through a periphery-to-brain crosstalk in an experimental model of AN; 2) investigating AN-induced morphological changes in different brain areas; 3) evaluating whether AN-induced molecular alterations and morphological changes contributes to cognitive impairments using cognitive behavioral test and 4) identifying the molecular scars that persist when body weight is restored, in an attempt to link motivated behavior, cognition and energy balance.

To verify this hypothesis, we employed the well-known animal model of AN, the activity-based anorexia (ABA) rat model, which consists of exposing adolescent female rats to the combination of food restriction and intense exercise, mimicking the hallmarks of AN pathology. At molecular level, we firstly focused our attention on specific brain areas that play crucial roles in the reward system, in processing feeding information, and in mediating high-level cognitive functions such as the NAc and the mPFC. We evaluated critical determinants of the glutamatergic system as the main mediator of the excitatory transmission in the CNS, and as a key regulator of motivation, executive functions, and actions, that, if altered, might be responsible for the reiteration of maladaptive responses to natural rewards, such as food.

Moreover, recent findings further pointed out a critical involvement of the neurotrophin BDNF not only in neuroplasticity and cognition but also in the homeostatic regulation of food intake, weight and energy expenditure, raising the interest for BDNF as a potential player in the crosstalk between periphery and brain that may underpin AN. Thus, we investigated whether food restriction coupled with intense exercise may activate a *muscle-to-brain* signal that arises from skeletal muscles and, via a PGC1α-FNDC5/Irisin mechanism, culminate in the brain modulating BDNF expression in the Hip, a brain area crucial for associative reward learning.

The final purpose of this multi-organ approach, combined with behavioral tests to evaluate cognitive performance and reward functionality in ABA animals, is to expand the understanding of the molecular and cellular basis of AN with the hope and the ambition to introduce novel scientific knowledge in the clinical setting.

3 Materials and methods

3.1 *Animals*

Adolescent female Sprague-Dawley rats were obtained from Charles River (Calco, Italy).
All animal procedures were carried out in accordance with the principles set out in the following laws and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008, by Ministry of Health); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) (Paxinos & Watson, 2013) and EU directives and guidelines (EEC Council Directive 2010/63/UE). All efforts were made to minimize animal suffering and to keep the lowest number of animals used: for ethical reasons, activity-based anorexia (ABA) rats were not allowed to lose more than 25% of their initial body weight. The experiments have been reported in compliance with the ARRIVE guidelines.

3.2 *Experimental design: the activity-based anorexia protocol*

Rodents arrived one week before the beginning of the experiment and were housed under a reversed 12 h light/dark cycle (light on 10.30 pm, light off 10.30 am). The animals were maintained under standard conditions of temperature (21±1°C) and humidity (50-60%) and fed with standard rat chow (ssniff Spezialdiäten GmbH, Soest, Germany) with tap water ad libitum. A maximum of two female siblings was taken from each litter in order to reduce "litter effects" (Chapman & Stern, 1978).
The ABA rat model was set up according to the literature (Carrera et al., 2014) with minor modifications.
For all the experiments, animals arrived at postnatal day (PND)24 and left undisturbed in grouped cages to habituate to the reversed light/dark cycle for at least one week before the beginning of the experimental procedures. Figure 11 show a schematic representation of the experimental paradigm (Fig 11).
At PND35 animals were individually housed in transparent cages lined with dust-free woodchips and randomly subdivided into four groups:

- [1] *control* (CTRL) group: sedentary + food ad libitum,

- [2] *food-restricted* (FR) group: sedentary + food restriction (food access limited for 2 h/day),
- [3] *exercise* (EXE) group: voluntary running activity + food ad libitum,
- [4] *activity-based anorexia* (ABA) group: voluntary running activity + food restriction (food limitation for 2 h/day).

EXE and ABA groups were allowed to perform voluntary running activity in home cages equipped with a mechanical activity wheel (activity wheel BIO-ACTIVW-R cage, Bioseb, France) properly furnished with an activity counter device.

At PND38, after three days of acclimation period to the new housing condition only FR and ABA groups were subjected to the caloric restriction paradigm with food limited for 2 h/day (free food 10.30 to 12.30 a.m.). During this time window of free access to food the wheel was preventively blocked to avoid the rats preferring to run rather than to eat.

At PND42, once the ABA animals reached the acute phase of the anorexic phenotype, half of the animals per group were sacrificed (PND42, first time point), while the remaining were preserved as sedentary, indeed they were placed back in classical home cages with food ad libitum to allow body weight recovery, for 7 days until PND49 (second time point).

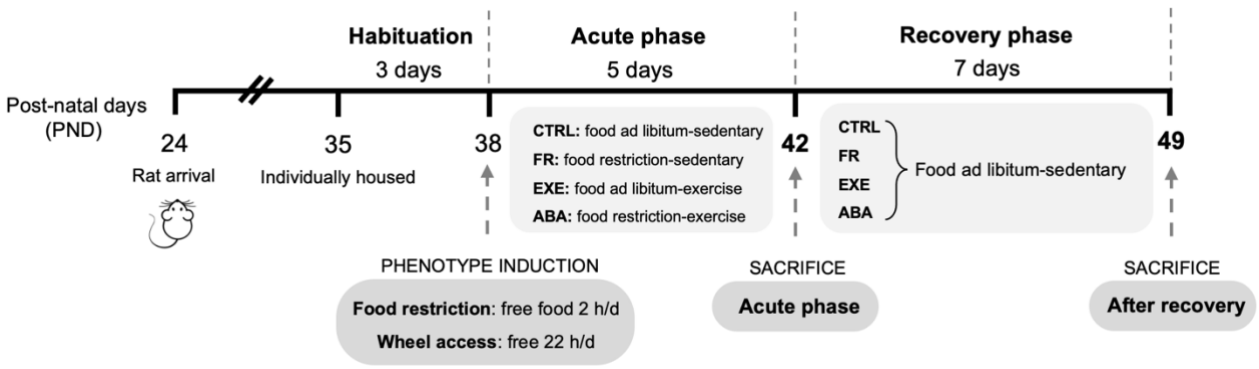
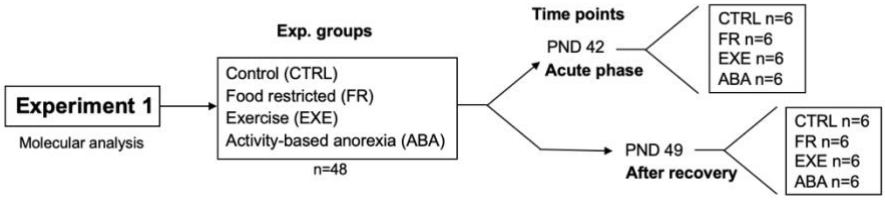


Figure 11 Schematic representation of the experimental paradigm performed in female adolescent rats to induce the activity-based anorexia (ABA) phenotype.

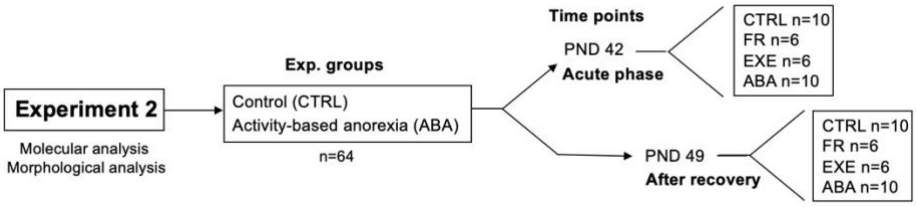
3.2.1 Experiments

Different sets of rats were used to set up the protocol and characterize the animal model, to perform molecular analysis and to conduct the cognitively demanding tests for memory performance evaluation.

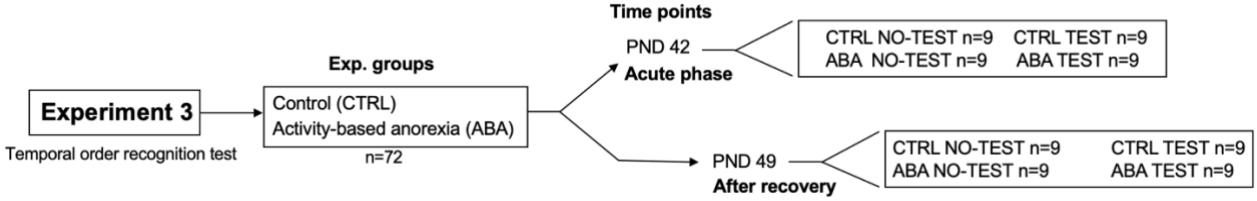
Experiment 1: behavioral characterization of the ABA model and molecular analysis n=48; 4 experimental groups for each time point



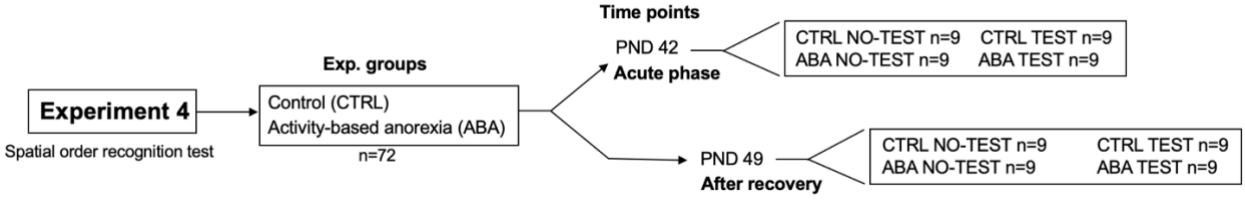
Experiment 2: molecular analysis and spine density and morphological analysis
n=64; 4 experimental groups for each time point for molecular analysis n=48; 2 experimental groups for each time point for morphological analysis n=16



Experiment 3: temporal memory performance evaluation
n =72; 4 experimental groups for each time point



Experiment 4: spatial memory performance evaluation
n =72; 4 experimental groups for each time point



For each of the above-mentioned experiments all the animals were carefully monitored: body weight, food intake and wheel running activity parameters were daily assessed, and to minimize disturbances each procedure occurred before the dark shift. Food intake was calculated as grams of given food at the beginning of the 2 h of food access – grams weighed at the end of the 2 h. Running wheel data were recorded, by means of a counter device and a monitoring software (BIO-ACTIVW-SOFT), at 30 minutes intervals for the entire duration of the experiments.

3.3 Behavioral tests

After the induction of the anorexic phenotype, animals belonging to *Experiment 3* and *4* (CTRL and ABA) were exposed to the Temporal Order Recognition Test (TOR) and the Spatial Order Recognition Test (SOR), respectively, to test rodents' ability to cope with temporal and spatial learning tasks in both the acute phase (PND42) and after a period of body-weight recovery (PND49).

The experimental apparatus used for the behavioral tests was a black open-field box (60x60x60 cm) made of Plexiglas, and the exploration time was taken by two independent investigators, blind to the experimental design.

Exploratory behavior was defined as the animal directing its nose toward the object at <2 cm of distance and sniffing, or snout directed to the object. Any subjects that failed to complete a minimum of 15 s exploration in the sample phase or 10 s of exploration in the test phase were excluded from the analysis.

3.3.1 Temporal order recognition test

This task, performed in *Experiment 3*, comprised two sample phases of 5 min duration each, with a delay in between of 1 h, and a final test phase of 5 min duration, performed 3 h after sample phase 2 (Fig 12). During sample phase 1 and 2, two different copies of objects were presented to the animals, adequately placed in two parallel corners of the squared arena. During the test, a single third copy of the objects from sample phase 1 and a single third copy of the objects from sample phase 2 were used. Both objects and their placement were counterbalanced.

During the test phase two different indexes were measured:

1) the recency discrimination index (D_I), that was calculated as the difference in time spent

by each animal exploring the object from sample phase 1 (Ob_1) compared with the object from sample phase 2 (Ob_2) divided by the total time spent exploring both objects (Ob_1+Ob_2) in the test period.

$$D_I = \frac{Ob_1 - Ob_2}{(Ob_1+Ob_2)}$$

2) the recognition index (R_I), that is considered as a measure of approach to both objects, was calculated as the time spent investigating the object presented in phase 1 (Ob_1) or the object presented in phase 2 (Ob_2) relative to the total object investigation time (Ob_1+Ob_2).

$$R_I = \frac{Ob_1 \text{ or } Ob_2}{(Ob_1+Ob_2)}$$

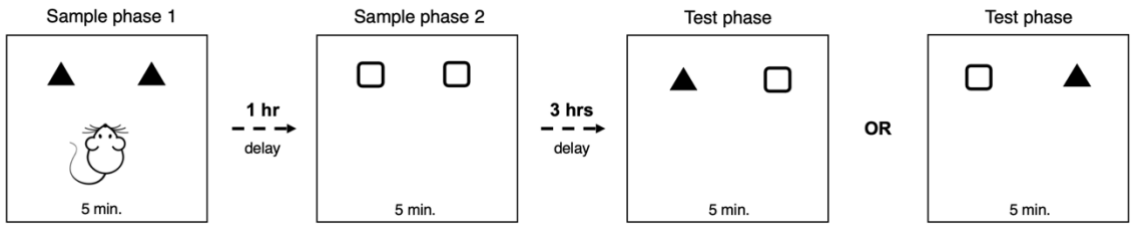


Figure 12 Schematic representation of the temporal order recognition (TOR) test performed in Experiment 3 in CTRL and ABA rats.

3.3.2 Spatial order recognition test

This task (Fig 13) was performed in *Experiment 4* (CTRL and ABA) and comprised one sample phase, and one test phase of 10 min duration, with a delay in between of 1 h. During the sample phase, two equal objects were presented to the animals, adequately placed in the top corners of the squared arena. During the test, the same two objects were presented, but one of them was displaced in the bottom-opposite corner of the arena, and as well as in the previous described task, all the objects and their placement were properly counterbalanced.

In this case the discrimination index (D_I) was calculated as well as for the TOR test (see above) with minor modifications: Ob_1 was considered the object not displaced, and the Ob_2 the object displaced in respect to the original position of the sample phase.

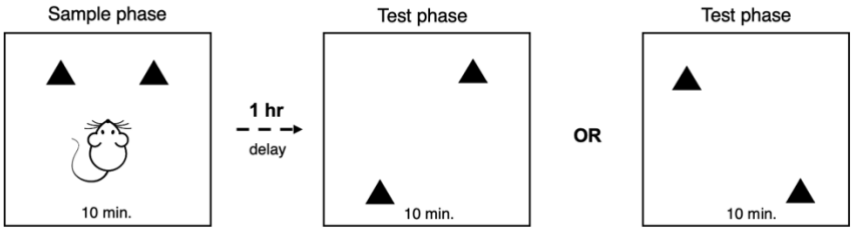


Figure 13 Schematic representation of the spatial order recognition (SOR) test performed in Experiment 4 in CTRL and ABA rats.

3.3.3 Locomotor activity

Locomotor activity was monitored both in *Experiment 3* and *4* during the test phase of both TOR and SOR tests to exclude animals with long periods of immobility and freezing and to set a baseline of movement among the different experimental groups. Locomotor activity was evaluated as number of passages (counts) among the four quadrants in which the arena was divided during test session.

Any subjects that failed to complete a minimum of 15 s exploration in the sample phase or 10 s of exploration in the test phase were excluded from the analysis. A total of 9 adolescent animals were removed from the behavioral studies because they failed to successfully explore the objects (more than 10 s) during the sample phase 1 or 2. Exploration time was taken by two independent investigators, blind to the experimental design.

3.4 *Molecular analysis*

Molecular analyses of gene and protein expression levels were performed on brain and muscle tissues of *Experiment 1* animals, that were immediately collected after decapitation, at two time points of sacrifice: at PND42, i.e. the acute phase of the pathology, and at PND49, i.e. after body weight recovery.

After decapitation, the medial prefrontal cortex (mPFC defined as Cg1, Cg3, and IL subregions), the Nucleus Accumbens (NAc defined as Shell and Core subregions), and the Hippocampus (Hip defined as CA1, CA3, GD) were freshly dissected from the whole brain, frozen on dry ice, and stored at −80°C. Brain regions were grossly dissected from 2 mm slices, indicatively following the coordinated of the Rat brain atlas of Paxinos and Watson (Paxinos & Watson, 2013) as follows:

- mPFC: from Bregma +5.64 mm to Bregma +3.72 mm (plates 5–9),
- NAc: from Bregma +2.76 mm to Bregma +0.84 mm, (plates 11–25),
- Hip: from Bregma -1.56 mm to Bregma -6.48 mm, (plates 46–89).

Soleus muscle tissues were entirely collected from right and left legs, immediately frozen on dry ice, and stored at -80°C.

3.4.1 RNA extracts preparation and Real-Time PCR analysis

Gene expression analysis was conducted on hippocampal and soleus mRNA extracts from *Experiment 1*.

Total RNA from Hip was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Milan, Italy) according to the manufacturer's instructions. Total RNA from soleus muscle was isolated with the RNeasy Fibrous Tissue Mini kit (Qiagen, Hilden, Germany) equipped with RNase free Mini Columns, according to the manufacturer's instructions.

The total RNA extracted was then quantified by Nanodrop spectrophotometric analysis. Following RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (Real time RT-PCR) to assess mRNA levels. In brief, an aliquot of each sample was treated with DNase (DNase I, RNase- freebuffer di MnCl2-Thermo Scientific) to avoid DNA contamination, RNA was analysed by TaqMan qRT-PCR instrument (CFX384

real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories), and samples were run in 384-well formats in triplicate as multiplexed reactions. Data were analysed with the comparative threshold cycle (ΔΔCt) method using 36B4 as the reference gene.

Gene expression analysis was performed in the Hip to evaluate *total Bdnf* gene expression while in the soleus to evaluate the expression of *Glut4* and *Cpt1β*. Primers and probes sequences were purchased from Eurofins MWG-Operon (See Table 5 for sequences).

Gene	Forward primer	Reverse primer	Probe
36b4	TCAGTGCCTCACTCCATCAT	AGGAAGGCCTTGACCTTTTC	TGGATACAAAAGGGTCCTGG
total Bdnf	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
Glut4 (Slc2a4)	ACAATGTCTTGGCTGTGCTG	TCCCACATACATAGGCACCA	GATACTCATTCTCGGACGGTTC
Cpt1β	TCTCAGCCTCTACGGCAAAT	TCTGCCCATGAGTGTTCTGT	GCACGGCAACTGCTATAACA

Table 5 Sequences of forward and reverse primers and probes used in Real-time PCR analyses (Eurofins MWG-Operon).

3.4.2 Protein extracts preparation and Western Blot analysis

Proteins from NAc, mPFC and Hip (*Experiment 1*) were homogenized in a teflon-glass potter using a cold buffer in the presence of a complete set of protease and phosphatase inhibitors cocktail.

In particular, the crude synaptosomal fraction was isolated from NAc and Hip, while the post-synaptic density fraction was extracted and purified from the mPFC.

Briefly, after the initial homogenization process an aliquot of each sample was kept and sonicated to be conserved as whole homogenate fraction, finally stored at -20°C for future molecular analysis. The remaining homogenate was centrifuged at 1000 g for 10 min obtaining a pellet (P1) corresponding to the nuclear fraction. The supernatant (S1) was then centrifuged at 9000g for 15 min to obtain a clarified fraction of cytosolic proteins (S2) and a pellet (P2). The P2 of mPFC extract, corresponding to a crude membrane fraction, was resuspended in a buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000 g for 1h. The resulting supernatant, referred as Triton X-100 soluble fraction (TSF), was stored at −20°C; the pellet, referred as postsynaptic density (PSD) or Triton X-100 insoluble fraction (TIF), was homogenized in a glass–glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at −20°C. The P2 obtained from NAc, and Hip was resuspended in a buffer containing 20 mM HEPES, 0.1 mM dithiothreitol, 0.1 mM EGTA, in presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail, and then stored at −20°C.

Total proteins from soleus muscle were isolated as follows. The total homogenate was obtained without sub-fractionation. The homogenization process was performed in RIPA buffer with mechanical disruption with TissueLyser disruptor (30 Hz, 30 sec, x3) and sonication (2 cycles). After sonication the samples were placed in ice for 15 min, and then centrifuged at 13000 g for 5 min at 4°C. In the last step the supernatant containing the enriched protein fraction was kept and stored at −20°C, while the pellet was discarded.

Total protein amount for all fractions of NAc, mPFC, Hip and Soleus was quantified through the Bradford Protein Assay, with bovine serum albumin as the calibration standard (Bio-Rad Laboratories, Italy). Calibration curve was run in duplicate, absorbance of the curves and of the samples was measured at 595 nm wavelength, and protein concentration of each sample was calculated related to the standard curve.

Equal amounts of proteins were run on home-made 8% polyacrylamide criterion gels, or on TGX precast gels or Stain free AnyKD gels (Bio-Rad Laboratories, Milan, Italy) depending on the targets of interest, under reducing conditions and then, electrophoretically transferred onto

nitrocellulose membrane (Bio-Rad Laboratories, Italy). Blots were blocked 1 h at room temperature with I-Block solution in TBS + 0.1% Tween-20 buffer, incubated with antibodies against the phosphorylated forms of the proteins, then stripped and reprobed with the antibodies against corresponding total protein. A table with specific antibodies conditions is provided below (Table 6).

Results from brain extracts were standardized to α-tubulin or β-actin control protein that were detected by evaluating the band density at 50 kDa or 43 kDa, respectively (α-tubulin: 1:20.000, Sigma; β-actin 1:15.000, Sigma). Results from muscle tissues extracts were standardized to the total protein content. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Gels were run two times each, and the results represent the average from two different runs. We used a correction factor to average the different gels: correction factor gel B = average of (OD protein of interest/OD control protein for each sample loaded in gel A)/(OD protein of interest/OD control protein for the same sample loaded in gel B) (Caffino et al., 2020).

Protein	Primary antibody	Secondary antibody
GluA1 (108 kDa) (Cell Signaling)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
GluA2 (108 kDa) (Cell Signaling)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
GluN1 (120 kDa) (Cell Signaling)	1:2000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
GluN2A (180 kDa) (Cell Signaling)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
GluN2B (180 kDa) (Cell Signaling)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
SAP97 (97 kDa) (Cell Signaling)	1:2000 iblock buffer O/N 4°C	1:2000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
SAP102 (102 kDa) (Cell Signaling)	1:2000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
GRIP (122 kDa) (Cell Signaling)	1:2000 iblock buffer O/N 4°C	1:3000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
PSD95 (95 kDa) (Cell Signaling)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
Neuroligin-1(94 kDa) (Synaptic System)	1:3000 iblock buffer O/N 4°C	1:2000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
N-cadherin (100 kDa) (Santa cruz)	1:2000 iblock buffer O/N 4°C	1:2000 iblock 1 hr RT anti-Mouse IgG HRP (Cell Signaling)
F-actin (42 kDa) (Abcam)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
BDNF (14 kDa) (Icosagen)	1:500 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
pTrkB (145 kDa) (NovusBio)	1:200 iblock buffer O/N 4°C	1:750 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
TrkB (145 kDa) (Cell Signaling)	1:750 iblock buffer O/N 4°C	1:750 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
Pgc1a (91 kDa) (Cell signalling)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
FNDC5 (23 kDa) (Novus Biotechnology)	1:1000 iblock buffer O/N 4°C	1:1000 iblock 1 hr RT anti-Rabbit IgG HRP (Cell Signaling)
$\beta-$***actin*** (43 kDa) (Sigma)	1:20.000 iblock buffer O/N 4°C	1:10.000 iblock 1 hr RT anti-Mouse IgG HRP (Sigma)
$\alpha-$***tubulin*** (50 kDa) (Sigma)	1:20.000 iblock buffer O/N 4°C	1:15.000 iblock 1 hr RT anti-Mouse IgG HRP (Sigma)

Table 6 Conditions of the antibodies used in western blot analysis (O/N: overnight; RT: room temperature)

3.4.3 Plasma collection and ELISA assay

Trunk blood from each rat was promptly collected after decapitation in tubes containing EDTA (0.5 M, pH=8). Plasma was separated by centrifugation (6500 g for 20 min) and irisin (Phoenix, EK-067-29) levels were determined by an enzyme-linked immunosorbent assay (ELISA) using commercial kits, according to the manufacturer's instructions.

3.5 *Dendritic spine labelling and morphological analyses*

Dendritic spine labelling and morphological classification of glutamatergic pyramidal neurons (layer V) of the mPFC and the Hip were carried out using a lipophilic membrane tracer in animals belonging to CTRL and ABA groups from *Experiment 2* (n=16) at both PND42 and PND49. Animals were deeply anesthetized and perfused with 0.1 M phosphate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB. Brains were removed from the skulls and were postfixed at 4 C in 4% PFA in PB for 40 min. 2-mm thick slices containing the brain region of interest have been dissected from the postfixed brains and stained with a lipophilic dye, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DilC18(3)) (Life Technologies). Brain sections were left overnight at room temperature in PB to allow the Dil to completely diffuse through labelled neurons. Then, sections were postfixed in 4% PFA for 40 min at 4°C, washed three times in PB and 150-μm thick coronal slices were prepared using a vibratome. The coronal slices were then mounted on slides, covered in fluoromount (Sigma-Aldrich), and analyzed on a Zeiss LSM-510 laser confocal microscope with 63X objective.
The number of neurons quantified for the mPFC, and the Hip was at least 25 for each experimental group (from each neuron, a different number of dendritic segments was analyzed); neurons analyzed were belonging to 8 hemispheres per group. Analysis of dendritic spine morphology was performed with Fiji software released by ImageJ software; for each protrusion, we measured spine length, head, and neck width, which was used to classify dendritic spines into three categories (thin, stubby, and mushroom) (Gardoni et al., 2012; Harris et al., 1992). Image acquisition and quantification were performed from an operator blind to the experimental groups.

3.6 *Statistical analyses*

Data were collected in individual animals (independent determinations) and are presented as means and standard errors. Body weight, food intake, distance travelled on the wheel, mean speed, maximum speed, brief- and long-exercise sequences were analyzed by two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's multiple comparisons test. Morphological and molecular changes were analyzed by two-way ANOVA, with physical activity (sedentary vs. exercise) and food intake (food ad libitum vs. food restriction) as independent variables. When dictated by relevant interaction terms, Tukey's multiple comparisons test was used to characterize differences among individual groups of rats. However, when no interaction between physical activity and food intake was observed, only the main effects were reported.

The recency discrimination index measured in the TOR and SOR test was analyzed using a two-way ANOVA, manipulation (control vs ABA) and time of sacrifice (PND42 acute phase vs PND49 after weight recovery). Fisher's least significant difference (LSD) test was used to characterize differences among individual groups of rats. However, when no interaction between manipulation and time of sacrifice was observed, only the main effects were reported.

Pearson's product-moment coefficients were calculated to study potential correlations between behavioral outcomes induced by the ABA procedure, related to the wheel-running activity, and molecular changes observed in the NAc crude membrane fraction and in the post-synaptic density of the mPFC of ABA rodents. Subjects were eliminated from the final dataset if their data deviated from the mean by 2 SDs. Prism 8.2.1 (GraphPad Software, Prism v8.2.1, San Diego, CA, USA) was used to analyze all the data. Significance for all tests was assumed at $p < 0.05$.

4 Results and discussion

4.1 *Activity-based anorexia dynamically dysregulates the glutamatergic synapse in the Nucleus Accumbens of female adolescent rats*

Mottarlini F, Bottan G, Tarenzi B, Colciago A, Fumagalli F, Caffino L.
Nutrients. 2020 Nov 28;12(12):3661. doi: 10.3390/nu12123661. PMID: 33260714; PMCID: PMC7760003.

Dieting and intense exercise, core symptoms of AN, are initially goal-directed behaviors driven by a strong weight-loss desire that afterword evolves to compulsiveness, leading patients to an *out-of-control* spiral that strongly characterize the vicious loop of AN (Steinglass & Walsh, 2016). Even though the causal-effect relationship between low caloric intake and exercise is unclear, this shift towards compulsiveness might involve changes in the Nucleus Accumbens (NAc) synaptic physiology, a brain region critically involved in integrating motivational and reward aspects of feeding behaviour (Kelley, 2004). In this brain area, the glutamatergic system homeostasis might be involved in food-related behavior, since it is a key mediator of enhanced motivation for addictive drug-seeking and incubation of drug craving (Scofield et al., 2016). Of note, both AN patients and ABA rodents exhibit an altered activation of accumbal pathways, revealing the presence of a dysfunctional mesocorticolimbic reward circuitry (Fladung et al., 2013; Foldi et al., 2017b). Taking into account the relevant role played by the glutamatergic system in the NAc mediating reward-related pathologies, such as drug addiction (Scofield et al., 2016), we sought to investigate whether alteration of the NAc glutamatergic plasticity may be involved in promoting starvation and hyperactivity-related compulsive behaviors in AN, a condition that indeed resemble addictive disorders. With this goal, we investigated the expression of AMPA and NMDA receptors subunits and their specific scaffolding proteins as critical determinants of the glutamatergic synapse, in the whole homogenate and in the crude membrane fraction to dissect the effects induced by the ABA model both on translation and localization of receptors at the synaptic site. All the analysis conducted in this study were performed at two time points (please refer to Materials and methods section, *Experiment 1*): immediately after the achievement of the anorexic phenotype (PND42), which mimic the acute phase of the pathology, and after 7 days of body weight recovery (PND49), a time point that may give us clues about the molecular scars still present, despite body weight recovery.

4.1.1 **Results**

4.1.1.1 Characterization of the activity-based anorexia rat model: food restriction elicited hyperactive running behavior

Body weight, food intake and wheel activity of each single rodent were monitored for the entire duration of the experiment (Experiment 1).

Regarding the body weight, from the first day of the experiment (PND31) to the first day of food restriction (PND38), CTRL, FR, EXE and ABA rodents grow physiologically, and, moreover, EXE and ABA body weight was not affected from the period of habituation to the wheel (PND35-38) during which both groups performed physical activity (Fig 14 a). After 24 h of food restriction, from PND39, FR and ABA rodents started to lose weight in comparison to CTRL and EXE groups that were not subjected to the caloric limitation. Interestingly, at PND42, ABA rodents showed a significant decrease of body weight also in comparison to FR group, even though both groups at the same time point ate a comparable quantity of food (Fig 14 b), highlighting the impact of physical exercise on body weight loss. At PND42 the recovery period started: animals were placed back in classical home cages without food limitation or wheel access for 7 days to allow body weight recovery. In this phase all rodents belonging to FR and ABA groups increased their food intake (Fig 14 b), thus recovering their body weight (Fig 14 a). Despite both groups were subjected to the same experimental conditions, and ate the same quantity of food, the ABA group required more days to completely recover their body weight at the level of CTRL animals, further suggesting that only the combination of food restriction and physical exercise induced prolonged effects on animals' recovery (See Table 9-10 for statistics).

Concerning wheel activity, EXE and ABA rodents showed a stable trend of activity on the wheel during the habituation period (PND35-38), both in terms of distance travelled and mean or maximum speed (Fig 15 a-c). After 22 h of food restriction (PND39), ABA rats started to increment the distance travelled in respect to the EXE group, whose activity remained constant over days, and reached statistical significance starting from PND40 (Fig 15 a). The quantitative increase observed in terms of distance travelled performed by the ABA rats was also paralleled by a qualitative boost of their activity: from PND41 they showed a significant increase of mean and maximum speed on the wheel (Fig 15 b, c), an index that point to the running activity of ABA rats as a compulsive behavior. Moreover, we observed that ABA rats entered the wheel to perform more long-term exercise sequences, measured as distance travelled >2 m each time they entered in the wheel, than EXE animals (Fig 15 d), while brief-term exercise sequences

(distance travelled <2 m each access on the wheel) were approximately the same in both groups (Fig 15 e) (See Table 9-10 for statistics).

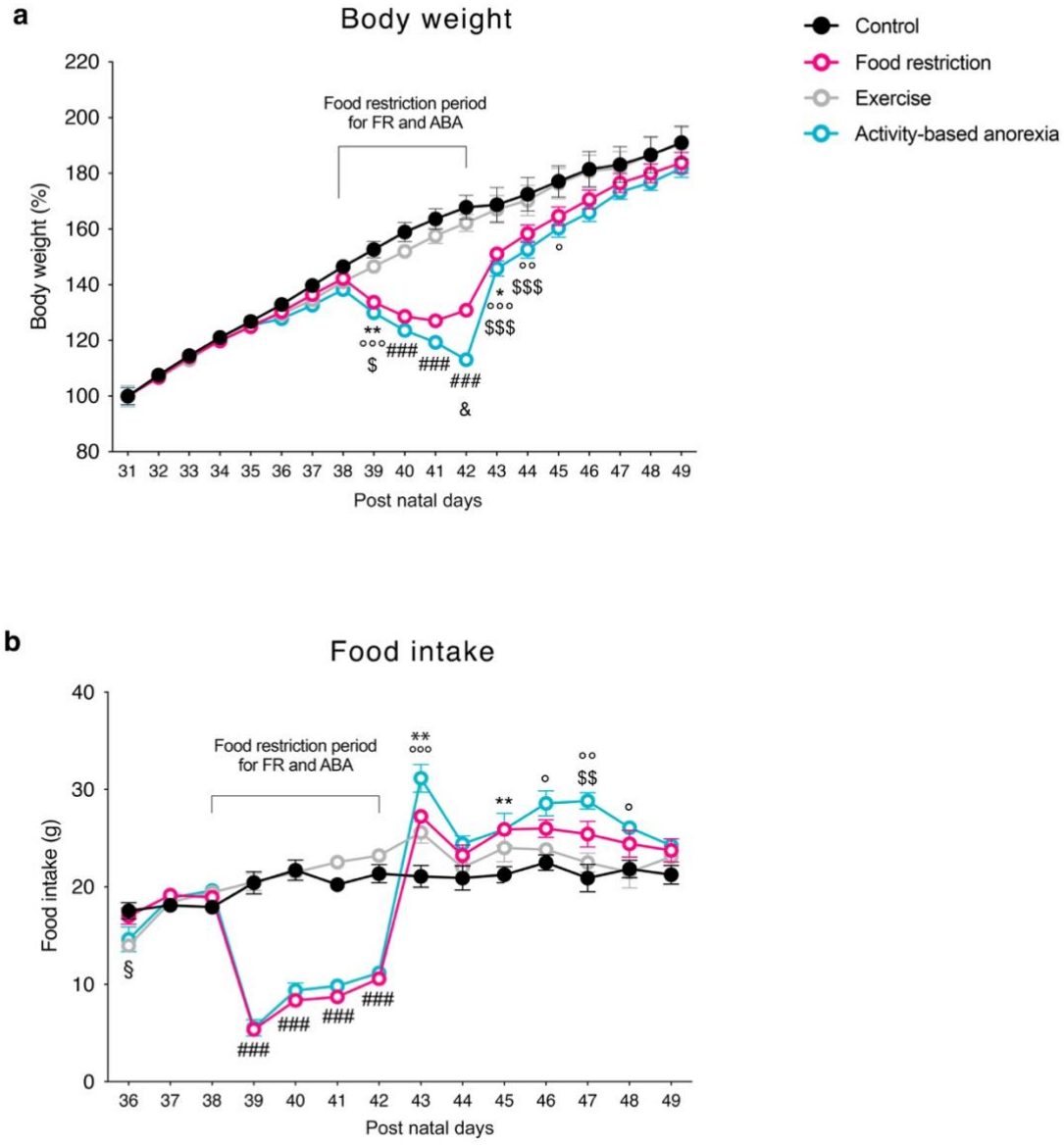


Figure 14 Average daily body weight **(a)** and food intake (**b**) measured in control (CTRL), food-restricted (FR), exercise (EXE) and activity-based anorexia (ABA) rats.

Results are presented as the mean ± SEM. $^{###}$ $p < 0.0001$ FR and ABA vs. CTRL and EXE; * $p < 0.05$, ** $p < 0.01$ CTRL vs. FR; $^{\circ}$ $p < 0.05$, $^{\circ\circ}$ $p < 0.01$, $^{\circ\circ\circ}$ $p < 0.001$ ABA vs. CTRL; $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ ABA vs. EXE; $^{\&}$ $p < 0.05$ FR vs. ABA (two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's multiple comparisons test).

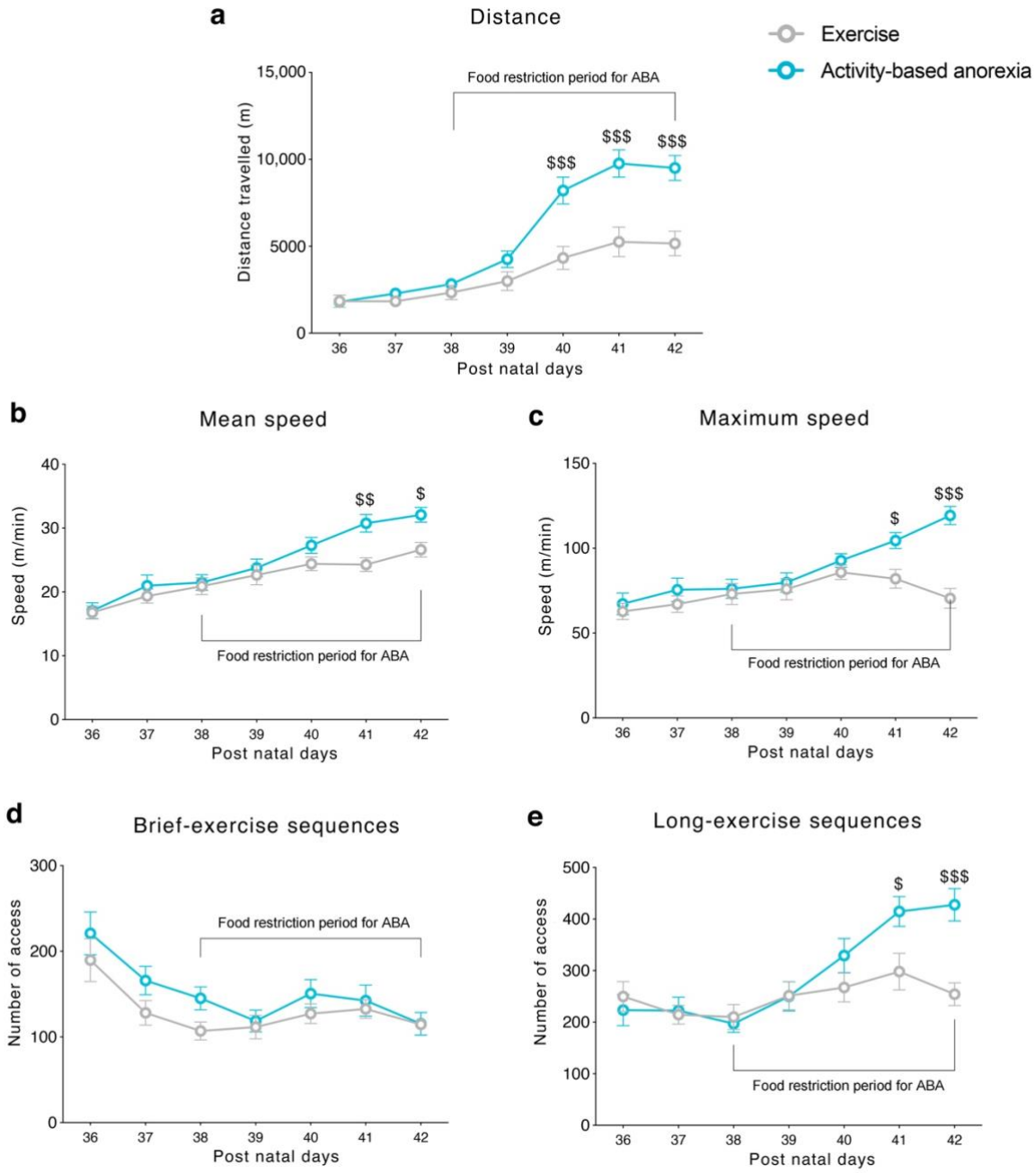


Figure 15 Running activity on the wheel in terms of (**a**) distance travelled in meters, (**b**) mean speed in meters/minute, (**c**) maximum speed in meters/minute, (**d**) brief- and (**e**) long-term exercise sequences expressed as the total number of accesses during which activity was lower or higher than 2 meters, respectively, exhibited by EXE and ABA rats. Results are presented as the mean ± SEM. $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ ABA vs. EXE (two-way ANOVA with repeated measures followed by Bonferroni's multiple comparisons test). EXE = exercise, ABA = activity-based anorexia.

4.1.1.2 Activity-based anorexia alters AMPA receptors and their scaffold proteins expression in the NAc

One of the primary mechanisms involved in the control of synaptic strength during synaptic plasticity is the alteration of AMPA receptors composition in the post-synaptic membrane (Diering & Huganir, 2018). It has been demonstrated that food restriction, as observed after long-term withdrawal from psychostimulant self-administration (Wolf, 2016), increases surface expression of GluA1-, but not GluA2-, containing AMPA receptors (Ouyang et al., 2017), suggesting that synaptic incorporation of GluA2-lacking Ca^{2+}-permeable AMPA receptors may underpin the development and persistence of starvation dependence.

Figure 16 show the GluA1/A2 ratio, which is widely accepted as an indirect index of maladaptive plasticity and incubation of drug craving (Wolf, 2016). No changes in AMPA receptor subunit composition were observed in the homogenate at both time points (Fig 16 a, c). Conversely, the GluA1/A2 ratio was markedly increased only in the crude membrane fraction of ABA rats in the acute phase of the pathology (Fig 16 b).

After a 7-days period of recovery, at PND49, the combination of food restriction and exercise increased membrane GluA1/A2 ratio (Fig 16 d), that instead was reduced when these two conditions were administered separately.

Since GluA1 and GluA2 are anchored to the membrane through specific scaffolding proteins that are also critical for synaptic localization of newly synthesized receptor towards dendritic spines and their functional activity (Naisbitt et al., 2000) we then investigated SAP97 and GRIP protein levels, the specific anchoring proteins of GluA1 and GluA2, respectively. ABA induction increased SAP97 levels in both homogenate (Fig 17 a) and membrane fraction (Fig 17 b) at PND42, whereas GRIP levels were increased only in the homogenate (Fig 18 a) and not in the membrane fraction (Fig 18 b). Conversely, both SAP97 and GRIP levels were reduced only in the membrane fraction of ABA rats after recovery (Fig 17 d and 18 d), whereas no changes were observed in the homogenate (SAP97: Fig 17 c; GRIP: Fig 18 c) (See Table 12-13 for statistics).

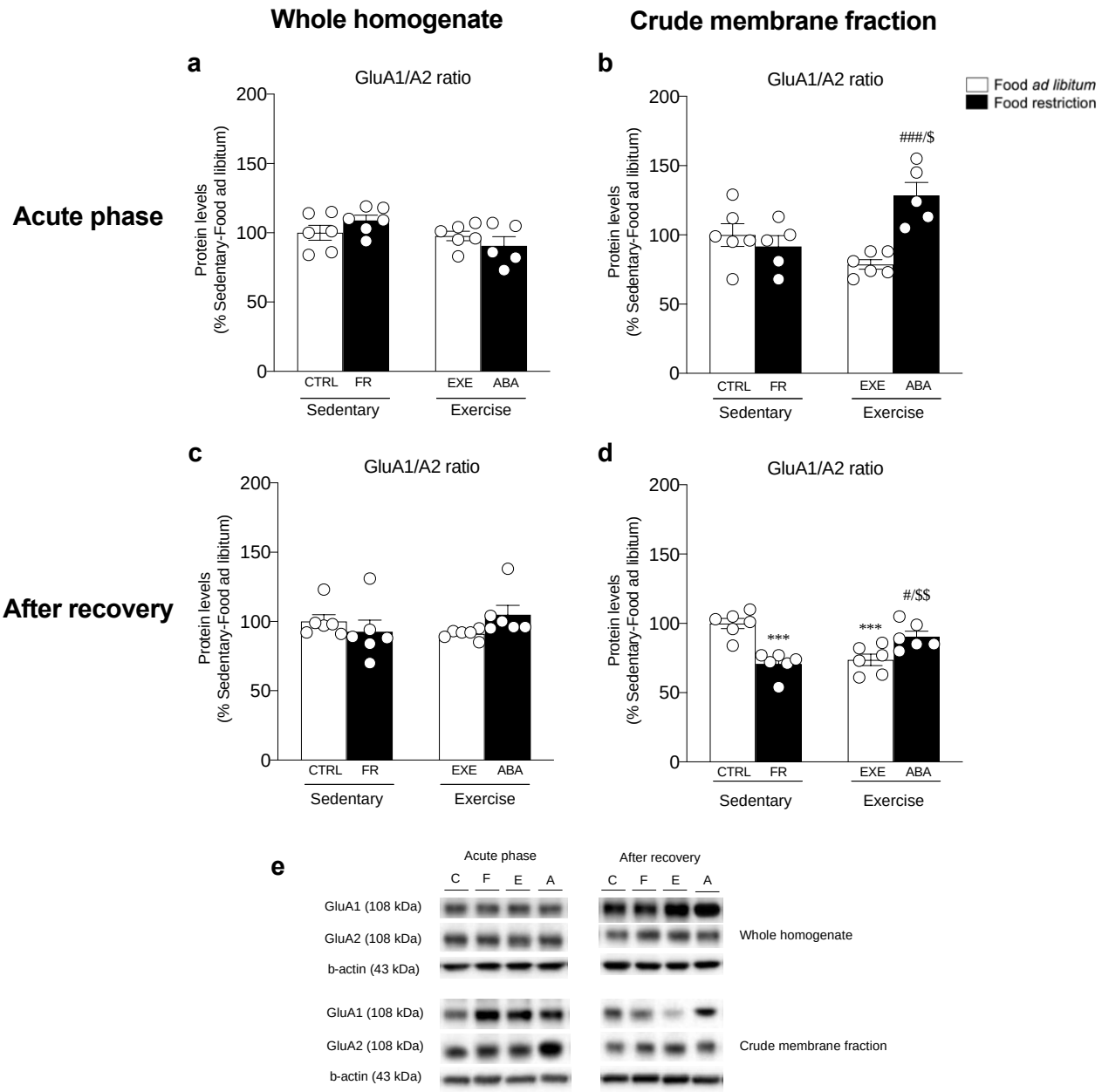


Figure 16. Effect of the ABA induction on the AMPA receptor subunits composition in the whole homogenate (left) and in the crude membrane fraction (right) of the NAc measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of GluA1 and GluA2 subunits expressed as GluA1/A2 ratio are shown in the (**a**) homogenate and (**b**) crude membrane fraction at PND42, and (**c**) in the homogenate and (**d**) crude membrane fraction at PND49. Results are expressed as percentages of controls and represent the mean ± SEM of five-six rats per. group. Panel (**e**) shows representative immunoblots for GluA1 and GluA2. $^{***}p < 0.001$ vs. Food ad libitum-sedentary; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. Food restriction-sedentary, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL (C) = control; FR (F) = food-restricted; EXE (E) = exercise; ABA (A) = activity-based anorexia.

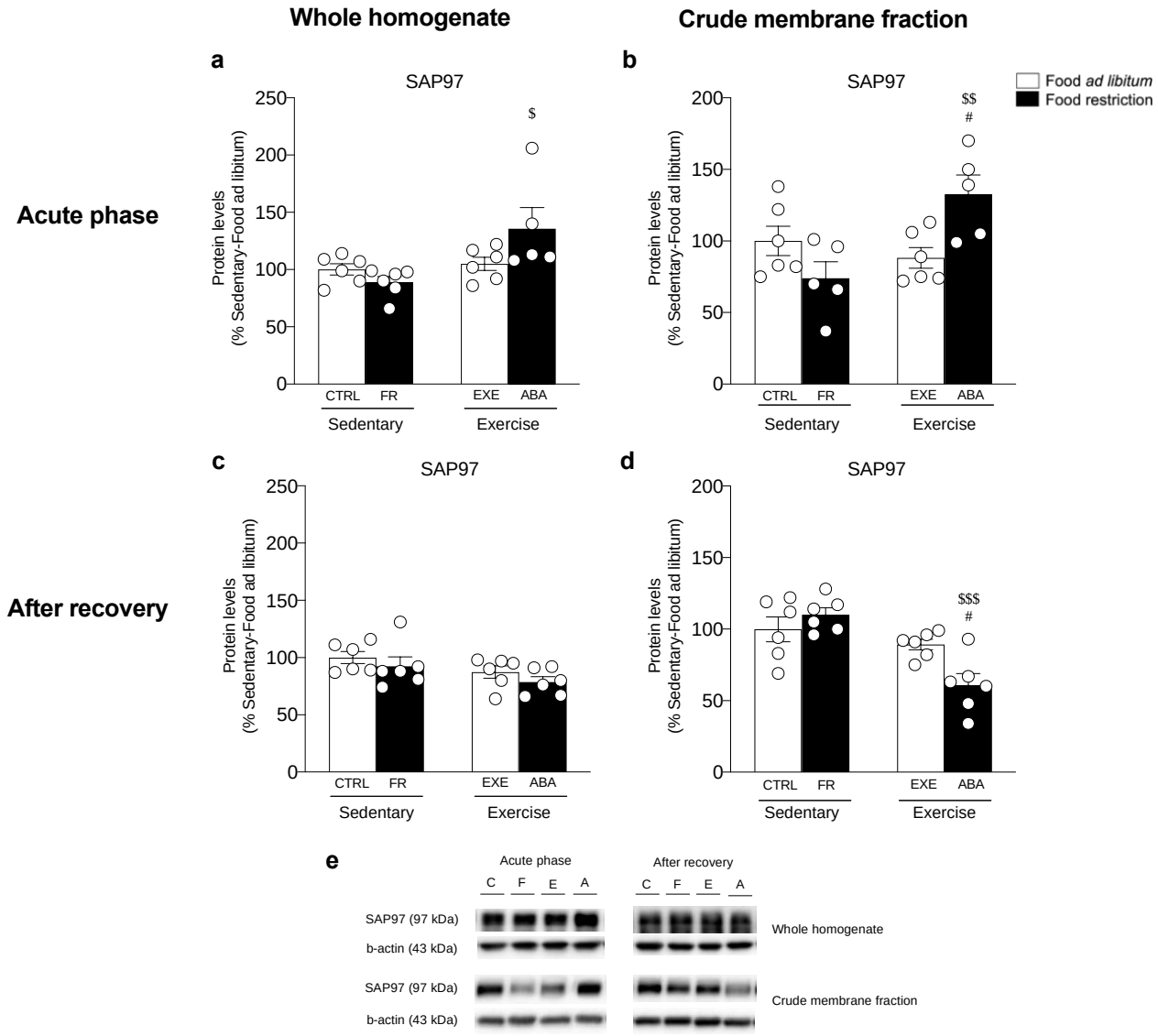


Figure 17. Effect of the ABA induction on SAP97 scaffolding protein expression in the whole homogenate (left) and in the crude membrane fraction (right) of the NAc measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of SAP97 are shown in the (**a**) homogenate and (**b**) crude membrane fraction at PND42, and in the (**c**) homogenate and (**d**) crude membrane fraction at PND49. Results are expressed as percentages of controls and represent the mean ± SEM of five-six rats per group. Panel (**e**) shows representative immunoblots for SAP97. $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ vs. Food restriction-sedentary, $^{\#}$ $p < 0.05$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL (C) = control; FR (F) = food-restricted; EXE (E) = exercise; ABA (A) = activity-based anorexia.

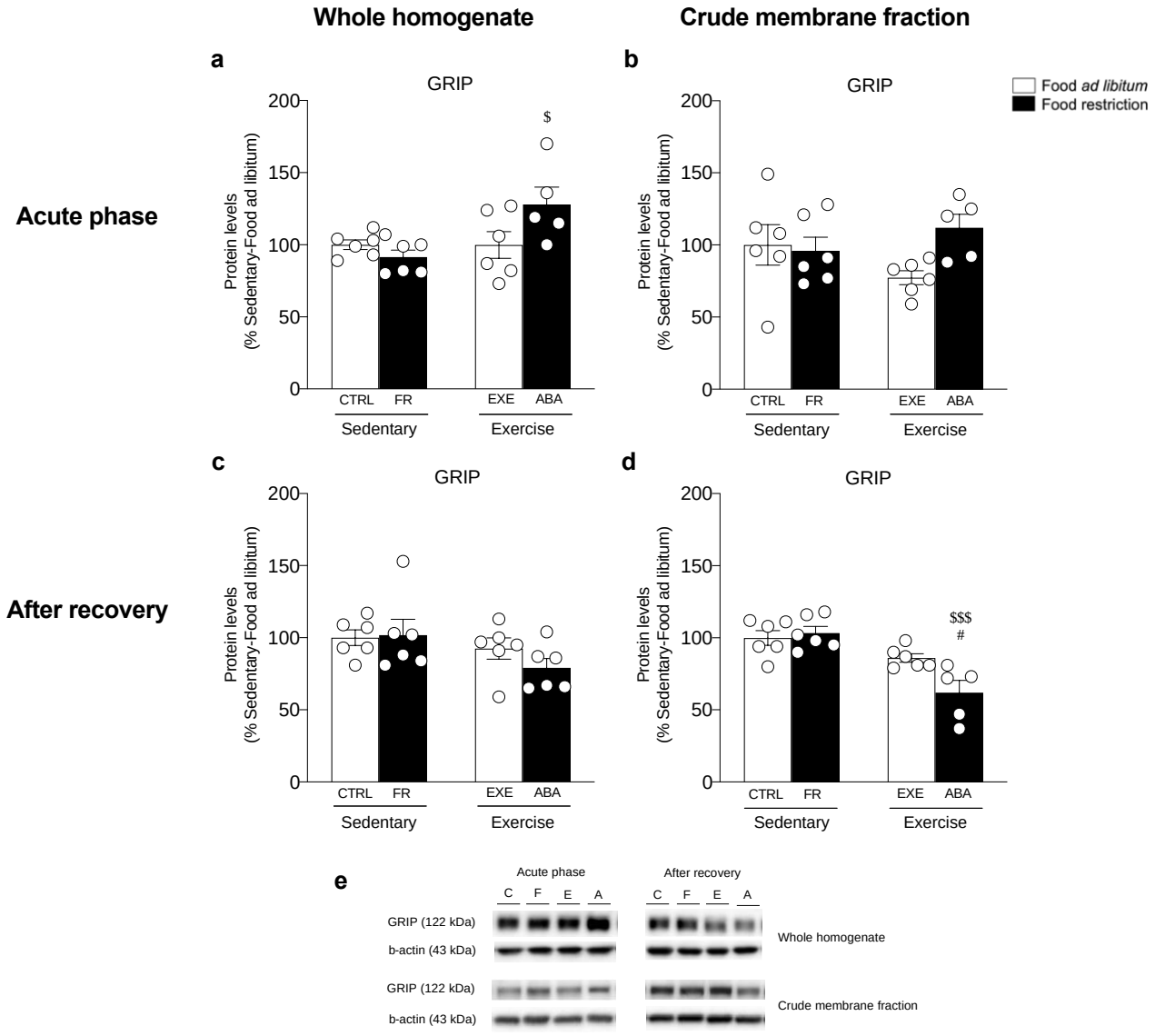


Figure 18. Effect of the ABA induction on GRIP scaffolding protein expression in the whole homogenate (left) and in the crude membrane fraction (right) of the NAc in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of GRIP are shown in the (**a**) homogenate and (**b**) crude membrane fraction at PND42, and in the (**c**) homogenate and (**d**) crude membrane fraction at PND49. Results are expressed as percentages of controls and represent the mean ± SEM of five-six rats per group. Panel (**e**) shows representative immunoblots for GRIP. $^{\$}$ $p < 0.05$, $^{\$\$\$}$ $p < 0.001$ vs. Food restriction-sedentary, $^{\#}$ $p < 0.05$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL (C) = control; FR (F) = food-restricted; EXE (E) = exercise; ABA (A) = activity-based anorexia.

4.1.1.3 Activity-based anorexia alters NMDA receptors subunits composition and their scaffolding protein expression in the NAc

NMDA receptors are glutamate-gated ion channels essential in neuroplasticity, and it has been shown that the different subunit composition of those receptors mediates the conversion of specific patterns of neuronal activity into long-term changes in synapse structure and function relevant for high-level cognitive processes and, in turn critical for various neurological and psychiatric disorders (Lau & Zukin, 2007; Traynelis et al., 2010). Of note, a switch between GluN2A- and GluN2B-containing NMDA receptors correlated with hunger-evoked exercise (Chen et al., 2017) and may alter the progression of the anorexic-induced phenotype.

Accordingly, we measured the GluN2A/2B ratio, that was reduced in the membrane fraction of FR rats whereas increased in ABA rats early after the induction of the phenotype (Fig 19 b) highlighting a diverse effect of food restriction in sedentary or hyperactive rats. On the contrary, the GluN2A/2B ratio was reduced only in the ABA recovered rats (Fig 19 d). No changes in NMDA receptor subunit composition were observed in the homogenate at both time points (Fig 19 a, c). In the acute phase of the pathology, no changes in PSD95 levels, a scaffolding protein of NMDA receptor and an index of postsynaptic density integrity (Chen et al., 2015; Vickers et al., 2006), were observed in both homogenate and membrane fraction (Fig 20 a and b, respectively). After recovery, PSD95 protein levels were increased in the homogenate (Fig 20 c) and reduced in the membrane fraction of ABA recovered rats (Fig 20 d). SAP102, another protein that anchor the NMDA receptor at the membrane (Muller et al., 1996), is reduced in the acute phase (Fig 21 b), while increased after recovery (Figure 8d), in the crude membrane fraction of ABA rats. No changes were observed in the homogenate at both time points (Fig 21 a, c) (See Table 12-13 for statistics).

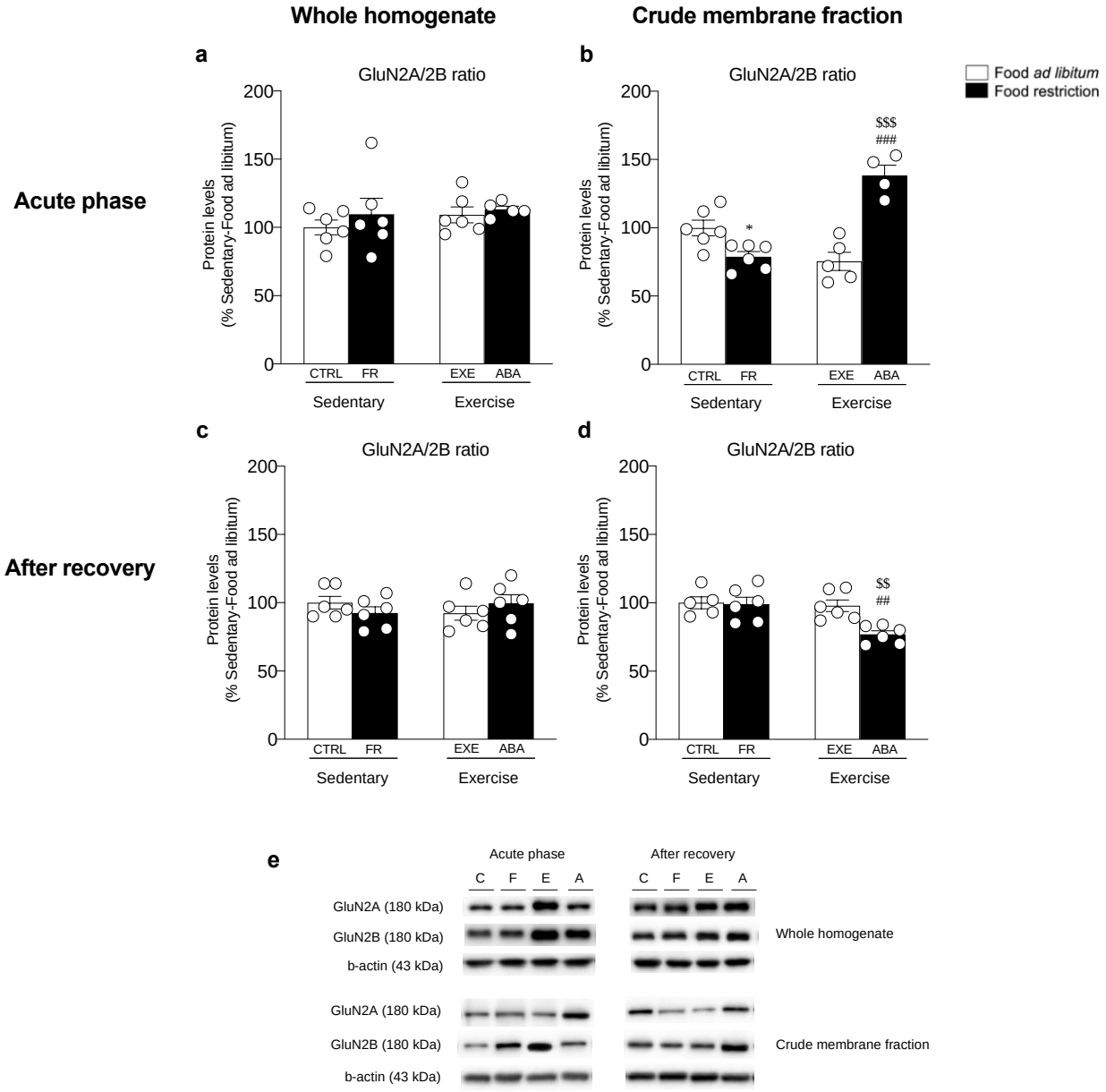


Figure 19. Effect of the ABA induction on the NMDA receptor subunits composition in the whole homogenate (left) and in the crude membrane fraction (right) of the NAc in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of GluN2A and GluN2B receptors expressed as GluN2A/2B ratio are shown in the (**a**) homogenate and (**b**) crude membrane fraction at PND42, and in the (**c**) homogenate and (**d**) crude membrane fraction at PND49. Results are expressed as percentages of controls and represent the mean ± SEM of five-six rats per group. Panel (**e**) shows representative immunoblots for GluN2A and GluN2B. * $p < 0.05$ vs. Food ad libitum-sedentary, $^{\$\$}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ vs. Food restriction-sedentary, $^{\#\#}$ $p < 0.01$, $^{\$\$\$}$ $p < 0.001$ vs. Food restriction-sedentary, $^{\#\#\#}$ $p < 0.001$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL (C) = control; FR (F) = food-restricted; EXE (E) = exercise; ABA (A) = activity-based anorexia.

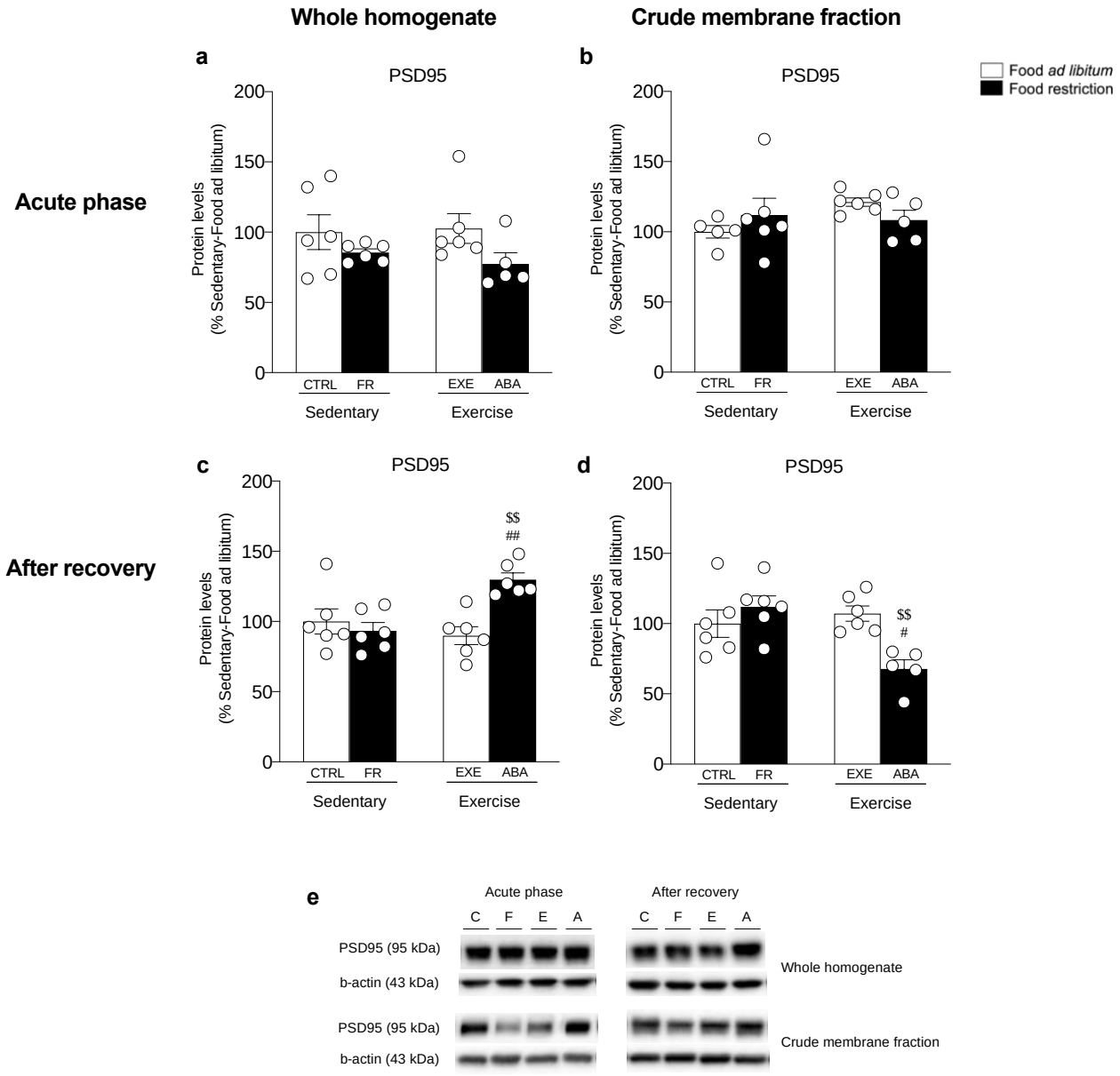


Figure 20. Effect of the ABA induction on PSD95 scaffolding protein expression in the whole homogenate (left) and in the crude membrane fraction (right) of the NAc in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of PSD95 are shown in the (**a**) homogenate and (**b**) crude membrane fraction at the PND42, and in (**c**) the homogenate and (**d**) crude membrane fraction at PND49. Results are expressed as percentages of controls and represent the mean ± SEM of five-***six*** rats per group. Panel (**e**) shows representative immunoblots for PSD95. $^{\$\$}$ $p < 0.01$ vs. Food restriction-sedentary, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL (C) = control; FR (F) = food-restricted; EXE (E) = exercise; ABA (A) = activity-based anorexia.

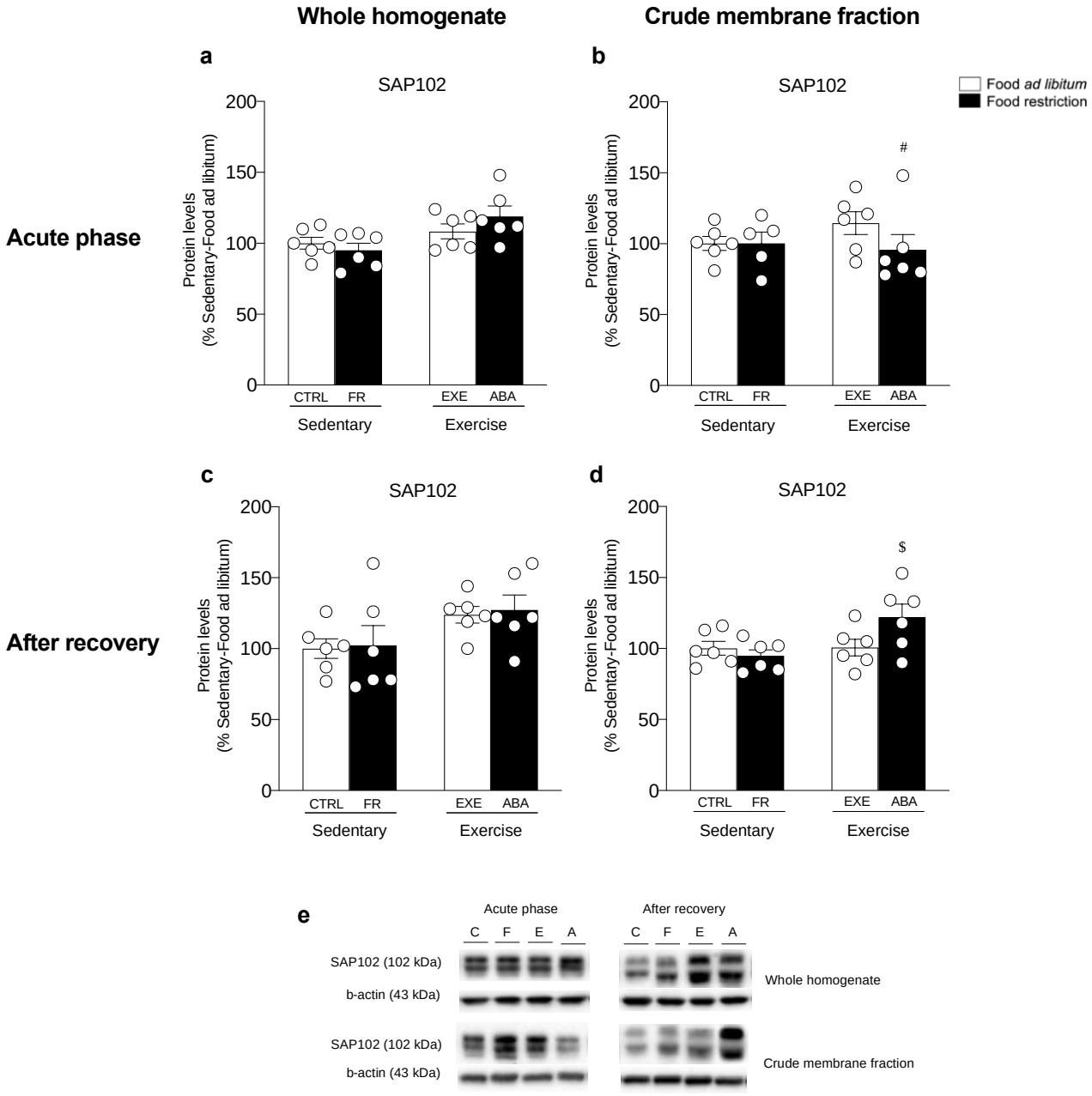


Figure 21. Effect of the ABA induction on SAP102 scaffolding protein expression in the whole homogenate (left) and in the crude membrane fraction (right) of the NAc in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of SAP102 are shown in the (**a**) homogenate and (**b**) crude membrane fraction at PND42, and in the (**c**) homogenate and (**d**) crude membrane fraction at PND49. Results are expressed as percentages of controls and represent the mean ± SEM of five-six rats per group. Panel (**e**) shows representative immunoblots for SAP102. $^{\$}$ $p < 0.05$ vs. Food restriction-sedentary, $^{\#}$ $p < 0.05$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL (C) = control; FR (F) = food-restricted; EXE (E) = exercise; ABA (A) = activity-based anorexia.

4.1.1.4 Food Restriction-Evoked Hyperactivity Correlated with GluA1/A2 Ratio

Pearson correlation analyses were run to determine the relationship between food restriction-induced hyperactivity and the herein investigated molecular markers in the NAc of ABA rats at both time points. In particular, Pearson's product–moment correlation was generated to correlate total distance travelled, mean and maximum speed on the wheel, long- and brief-exercise sequences with molecular results obtained from GluA1/A2, GluN2A/2B ratio and their scaffolding proteins analysis in the membrane fraction. As shown in Figure 22, GluA1/A2 ratio positively correlated with total distance travelled (Fig 22 a), mean speed (Fig 22 b) and long-exercise sequences (Fig 22 c) whereas PSD95 levels negatively correlated with maximum speed (Fig 22 e) and long-exercise sequences (Fig 22 d).

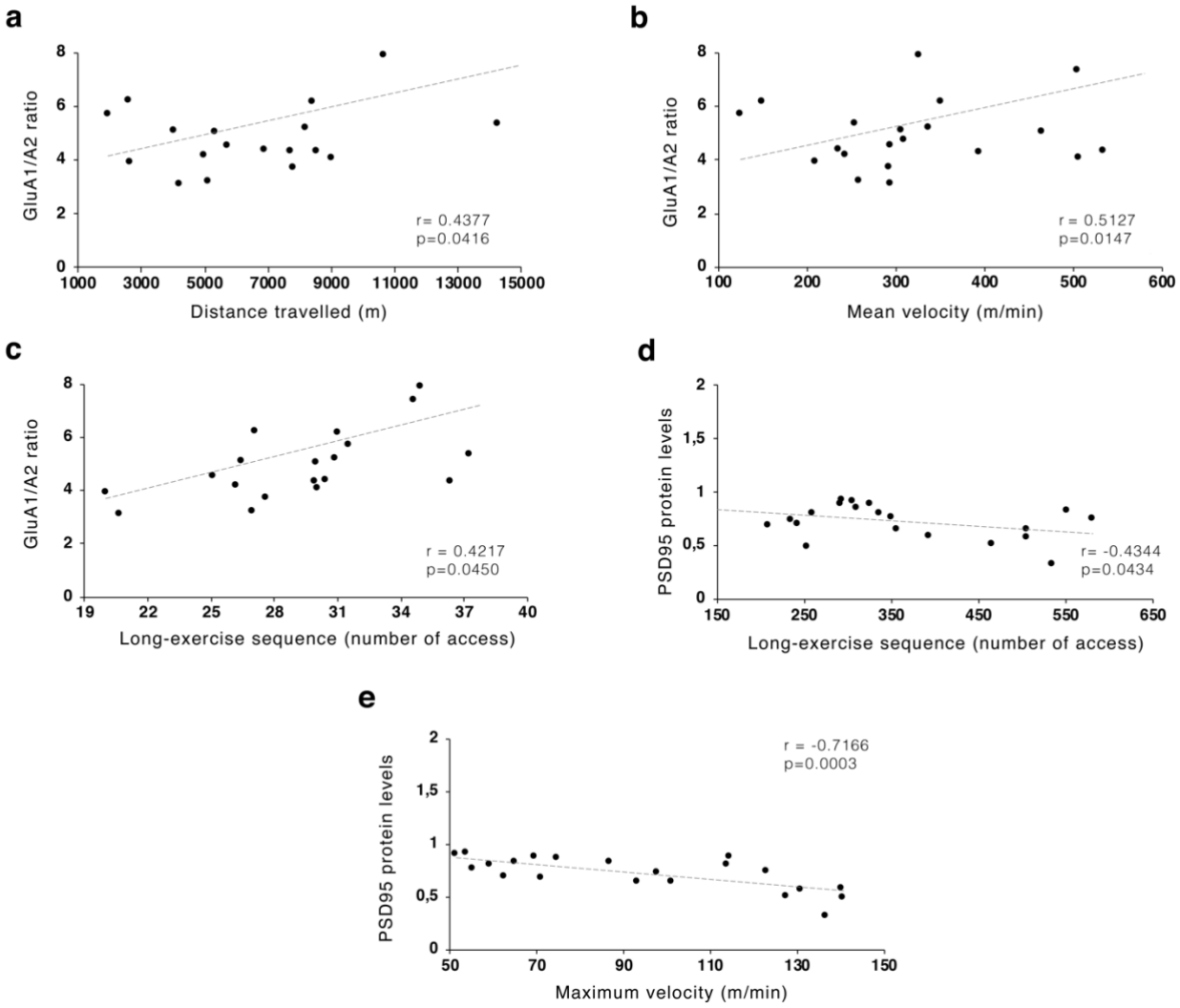


Figure 22. Pearson's product–moment correlation (*r*) analyses between GluA1/A2 ratio and (**a**) distance travelled, (**b**) mean speed and (**c**) long-exercise sequences and between PSD95 and (**d**) long-exercise sequences and (**e**) maximum speed of ABA and EXE rats. EXE = exercise; ABA = activity-based anorexia.

4.1.2 **Discussion**

In our first study on AN, taking advantage of the ABA model, we demonstrate that adolescent female rats exposed to a food restriction paradigm in concomitance with the possibility to perform physical exercise develop hyperactive behaviors, as shown by the exponential increase of distance travelled on the wheel by ABA rats. In fact, unlike EXE animals that maintained a voluntary and stable activity during the entire experiment, when food restriction began, wheel activity of ABA rats constantly increased over days, as expected in this model (Chen et al., 2020; Scherma et al., 2017). Of note, this food restriction-evoked hyperactivity gave rise to compulsive behaviors, as demonstrated by increased number of voluntary entries in the wheel to perform long-term exercise sequences, and by the boost of mean and maximum speed during wheel activity of ABA rats in respect to the EXE group, parameters indicative of a strong motivation to run despite the severe emaciation. At the end of the recovery period, body weight of both FR and ABA rats were restored back to control levels; however, ABA rats, despite a higher food intake compared to FR rats, took more time to restore it, underlying the strenuous and persistent impact of the intense physical activity in a condition of low food intake. These results confirm that low caloric intake drives animals toward a high level of physical activity, and moreover the combination of these two conditions induces animals to develop the anorexic phenotype, further validating the ABA model as a tool able to reproduce the hallmarks of anorexia nervosa (high physical exercise, low body weight). Despite the ABA model does not fully address the complex aspects of AN in humans, it gives us a readout of the main symptoms of anorexic subjects, allowing us to investigate the neurobiological underpinning of AN-induced hyperactive behavior. Given the impact that intense and compulsive physical exercise have on the full recovery of anorexic patients (Strober et al., 1997) and on their risk of relapse, the comprehension of the molecular mechanisms underlying this AN-induced feature is fundamental to better understand how AN develops and is maintained (Taranis & Meyer, 2011).

From a molecular point of view, our experiments revealed that the glutamate system is dysregulated in the NAc of ABA rats and the reorganization of the glutamate synapse correlates with ABA-induced hyperactive behavior. In particular, the induction of the ABA phenotype, but not food restriction or exercise alone, enhanced the GluA1/A2 protein ratio in the membrane, an index of GluA2-lacking Ca^{2+}-permeable AMPA (CP-AMPA) receptors formation (Reimers et al., 2011). This effect was paralleled by increased expression (whole homogenate) and retention in the membrane (crude membrane fraction) of SAP97, the

scaffolding protein pivotal for synaptic localization of GluA1 subunits of AMPA receptor (Kim et al., 2005a). Interestingly, the levels of the GluA2 specific scaffolding protein GRIP were increased in the whole homogenate, but its localization at the membrane was not affected, further indicating the presence and the increased stability of GluA1-, but not GluA2-containing, CP-AMPA receptors at the membrane. Since increased membrane insertion of CP-AMPA receptors may mediate excitotoxicity (Liu & Zukin, 2007), redirect the synapse functionality versus immature synapse (Bellone & Luscher, 2012), as well as promoting incubation of drug craving (Wolf, 2016), the alterations of the glutamate system herein observed in the NAc of ABA rats might influence synaptic transmission, ultimately driving maladaptive reward-related behaviors typical of AN patients (Bergh & Sodersten, 1996; Zink & Weinberger, 2010). The increase in GluA1/A2 ratio observed in the acute phase of the anorexic phenotype persisted even after 7 days of recovery, highlighting the presence of CP-AMPA receptors in the NAc as long-lasting signatures derived from the ABA condition. Of note, this increase was not accompanied by enhanced expression of the anchoring proteins SAP97 and GRIP, thereby suggesting a reduced AMPA receptor stability. Akin to previous data showing that food restriction alters NAc synaptic transmission via increased surface expression of GluA1, but not GluA2, subunit (Ouyang et al., 2017), our data add complexity as well as specificity to the herein shown reorganization of the AMPA receptor subunit demonstrating that also the synaptic machinery that might anchor these receptors in the membrane is persistently modified by ABA induction.

In concomitance with AMPA receptor subunits reorganization, the ABA phenotype increased the NMDA GluN2A/2B subunit ratio at the synaptic site in the acute phase, while such ratio is instead reduced after body weight recovery. Similar to AMPA receptors, the lack of effects in the whole homogenate implies that the combination of food restriction and hyperactivity has not influenced the translation of the glutamate receptors but, rather, their subunit reorganization and synaptic retention. It is well known that during neonatal development the GluN2A subunit expression gradually increases while GluN2B progressively decreases, a developmental switch that drives changes in synaptic transmission leading to mature synapse formation (Monyer et al., 1994). Moreover, experiences, such as addictive drug exposure (Yuan et al., 2013) or in this case ABA induction, are likely to reverse this switch toward GluN2B-containing NMDA receptors, thus re-opening a critical period of vulnerability and re-programming the accumbal excitatory synapse toward immature or silent synapse. Nonetheless, considering that the NMDA receptor-dependent plasticity is required for learning association between environmental cues and reward-related behaviors (Vega-Villar et al., 2019), this NMDA

subunits phasic plasticity observed in the NAc of ABA rats suggests that the consequent potential alteration in neurotransmission might be a signature of the ABA-induced vulnerability. In the acute phase of the pathology, despite the increase in GluN2A/2B ratio in the membrane fraction both anchoring proteins specific for NMDA receptor, SAP102 and PSD95, were reduced, thus suggesting NMDA receptor instability, and altered NMDA-dependent neurotransmission in ABA rats. In addition, the reduced expression of PSD95, a postsynaptic protein required for normal synaptic transmission of both AMPA and NMDA receptors (Vickers et al., 2006), in the membrane fraction, but not in the whole homogenate, further supports the hypothesis of impaired synaptic plasticity in the NAc of ABA rats. The reduced GluN2A/2B ratio observed despite body weight recovery might be considered as an index of vulnerability trait of ABA rats since it has been demonstrated that GluN2A knockout mice display an increased baseline physical activity and, when subjected to the ABA model, these mice lost significantly more weight than their wild-type littermates (Robinette et al., 2020). Moreover, our data are in line with the previously observed correlation between ABA severity and increased GluN2B-containing NMDA receptor observed in the postsynaptic terminal of the hippocampus (Chen et al., 2017).

Taken together, we demonstrate that low caloric intake is sufficient to compulsively increase physical activity in female adolescent rats, a core feature of AN pathology. This rapid escalation of maladaptive behaviors correlates with glutamate receptor subunit redistribution and, ultimately, to an altered molecular composition of the glutamatergic synapse in the NAc. From a neurobiological point of view, our findings, showing a switch toward GluA2-lacking and GluN2B-containing receptors, are reminiscent of the neuroadaptive changes of the glutamate synapse that are usually seen after chronic exposure to drugs of abuse (Liu & Zukin, 2007; Scofield et al., 2016). In fact, as observed for drugs of abuse, we hypothesized that ABA induction via reorganization of the glutamatergic synapse might re-open a critical period of vulnerability, during which changes in functional properties of glutamate receptors might influence synaptic transmission, remodel reward-related neurocircuitry and alter food- and exercise-related behavior. Our data, in line with the hypothesis that the imbalance between cognitive and reward circuitries likely interferes with motivation for treatment and ability to learn from experience (Milton et al., 2020), suggest that pharmacotherapies designed to manipulate the glutamate system might enable ill individuals to stop the vicious cycle of the disease.

4.2 *Cortical reorganization of the glutamate synapse in the activity-based anorexia rat model: impact on cognition*

Manuscript under revision
Mottarlini F, Bottan G, Targa, G, Tarenzi B, Fumagalli F, Caffino L.

The medial prefrontal cortex (mPFC) is a crucial brain region that mediates cognitive processes, emotion, motivation, and reward (Xu et al., 2019) integrating information from numerous areas and converging updated sensory and limbic inputs to output structures, promoting goal-directed behaviours through cortico-striatal loops (Tzschentke, 2000; Tzschentke & Schmidt, 2000). Despite the neuronal basis of AN is likely multifactorial, the failure to adapt to feeding signals, the generation of food-avoidance and altered expectancies to food are, at least in part, related to mPFC dysfunction. Notably, mPFC activation is consistently altered in AN patients performing the Wisconsin Card Sorting Test (Sato et al., 2013) or challenged with a Go/NoGo task (Lock et al., 2011). Besides, such patients also show aberrant excitability of the mPFC following different food-stimuli tests, an effect partially conserved even after a long period of recovery. These data highlight an altered reward-related response to a pleasurable stimulus in AN patients, pointing to dysfunctions in food information processing (Ehrlich et al., 2015; Kullmann et al., 2014; Sanders et al., 2015). Of note, these findings resemble preclinical observations in the ABA model: ABA rats showed deficits in performing reversal learning tasks, thus involving cortical abnormalities (Allen et al., 2017).
Thus, based on the hypothesis that malnutrition-induced impairment of brain morphology and connectivity may have a role in the behavioral phenotype of AN, the main goal of this study was to evaluate whether the combination of food restriction and intense exercise induces structural remodeling together with alterations in the glutamatergic signaling in the mPFC leading, in turn, to dysfunctional cognitive functions, such as the temporal memory processes, that might trigger, and feed, the motivational loop underlying AN (Lamanna et al., 2019).

4.2.1 **Results**

As described in the abovementioned chapter (**4.1**) regarding the phenotypic characterization of the ABA model (*Experiment 1*), adolescent female rats exposed to the combination of caloric restriction and physical exercise develop anorexic behaviors. We exposed another batch of rats to the ABA protocol (*Experiment 2*), and we observed that ABA rats progressively reduced body weight, at PND42 significantly more than FR rats (Fig 23 a), despite similar food intake (Fig 23 b). ABA rats progressively increased their running activity on the wheel, while EXE animals maintained a stable activity (Fig 23 c).

Of note, Pearson correlation analyses showed a negative correlation between the distance travelled on the last day of ABA induction (PND42) and body weight of both ABA and EXE rats (Fig 23 d), highlighting the inverse relationship between these two variables: as the distance travelled increases the body weight decreases (See Table 14 for statistics).

These results also attest a high reproducibility of the ABA model, since the data obtained from two different experiments (*Experiment 1* and *Experiment 2*) were strongly comparable between each other.

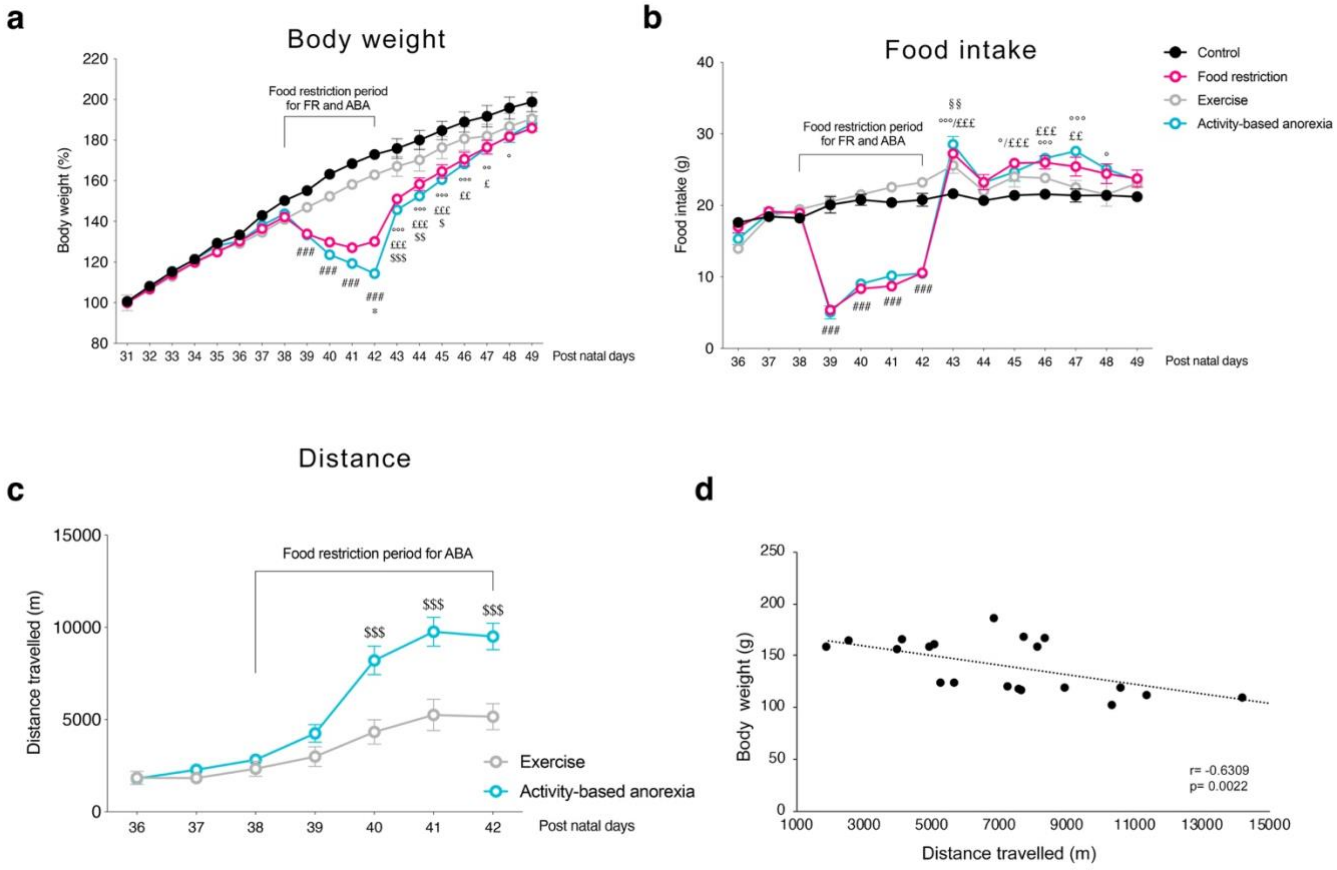


Figure 23. Average daily body weight **(a)** and food intake (**b**) measured in control (CTRL), food-restricted (FR), exercise (EXE) and ABA rats, and total daily distance travelled **(c)** in EXE and ABA rats. Panel **(d)** shows the Pearson's product–moment correlation (*r*) analyses between body weight and distance travelled at PND42 of ABA and EXE rats.

Results are presented as the mean ± SEM. $^{###}$p<0.0001 FR and ABA vs CTRL and EXE; *p<0.05 ABA vs FR; $^{°}$p<0.05, $^{°°}$p<0.01, $^{°°°}$p<0.001 ABA vs CTRL; $^{£}$p<0.05, $^{££}$p<0.01, $^{£££}$p<0.001 FR vs CTRL; $^{$}$p<0.05, $^{$$}$p<0.01, $^{$$$}$p<0.001 ABA vs EXE, §§p<0.01 CTRL vs EXE (Two-way ANOVA with repeated measures followed by Bonferroni's multiple comparisons test). FR = food-restricted; ABA = activity-based anorexia.

4.2.1.1 Activity-based anorexia induction dysregulates the molecular composition of the glutamate synapse in the mPFC

To determine whether ABA induction alters the cortical glutamate synapse homeostasis, we examined the expression of the main glutamate AMPA and NMDA receptors, their scaffolding proteins, and markers of structural plasticity in the mPFC postsynaptic density (PSD) of ABA rats in the acute phase of the disorder and after a 7-days recovery period.

ABA rats showed reduced levels of GluN1 (Fig 24 a), the obligatory subunit of NMDA receptor, in the acute phase of the pathology while the expression of GluN2A and GluN2B were reduced only in recovered ABA rats (Fig 24 b and 24 c, respectively). Interestingly food restriction and exercise per sé reduced only GluN2A and GluN2B in the postsynaptic density of the mPFC at PND42 (Fig 24 b and 24 c, respectively). Regarding the analysis of AMPA receptor subunits in the PSD of mPFC, ABA rats showed reduced levels of GluA1 (Fig 25 a) only in recovered ABA rats, while GluA2 levels were reduced in the acute phase of the pathology (Fig 25 b). The lone exercise increased GluA2 levels at PND42 whereas one week later, at PND49, its levels were reduced (Fig 25 b).

Akin to NMDA and AMPA receptor subunits, the specific scaffolding protein of NMDA receptors SAP102 (Fig 26 a), the specific scaffolding protein of AMPA receptors, SAP97 (Fig 26 b) and PSD95 (Fig 26 c) levels were reduced only in the cortical PSD of recovered ABA rats. Next, we evaluated the expression of filamentous F-Actin, the major cytoskeletal component of dendritic spines (Hotulainen & Hoogenraad, 2010), of neuroligin-1, a postsynaptic cell-adhesion molecule at the excitatory synapse involved in synaptic transmission (Luo et al., 2020), and of neuronal N-cadherin, a cell-cell adhesion protein, which are all proteins involved in maintaining the structural integrity of the synapse. F-actin is differently altered depending on the phase of the pathology analyzed. In fact, weight loss only in ABA rats reduced F-actin levels in the acute phase of the pathology (Fig 26 d), while body weight recovery markedly increased them. Differently from F-actin, the neuroligin-1 levels were reduced in ABA rats despite weight recovery (Fig 26 e). Moreover, neuroligin-1 levels were increased as a consequence of development. As for F-actin, N-cadherin levels were increased only in ABA recovered rats (Fig 26 f). Food restriction and exercise per sé did not produce any change in the structural markers herein analyzed (Fig 26 d-f), further suggesting that the composition and structure of the glutamate synapse is specifically altered by the combination of low food intake and hyperactivity. Pearson correlation analyses were run to determine the relationship between ABA induction and the herein investigated molecular

markers in the mPFC of ABA and EXE rats at both time points. As shown in figure 24 and 25, GluN1 (Fig 24 d) and GluA1 (Fig 25 d) negatively correlated with the distance travelled in the last day of ABA induction (PND42).

The analysis on the molecular composition of the glutamate synapse was extended to the cortical whole homogenate, in order to dissect the effect of the anorexic phenotype on glutamatergic markers translation from their availability at synaptic sites (Table 7). Interestingly, in the whole homogenate of the mPFC, exercise per se increased the expression of GluN1, GluN2B, GluN2A, GluA1, GluA2, SAP97, Neuroligin 1, whereas the induction of the anorexic phenotype reduced the protein expression of GluN2B, GluN2A, GluA1, GluA2, SAP97, F-actin and N-cadherin only in the acute phase of the pathology. Recovery of body weight did not produce any change in the translation of the main glutamate receptors, their specific scaffolding proteins, and markers of structural plasticity (See Table 15-16-17 for statistics).

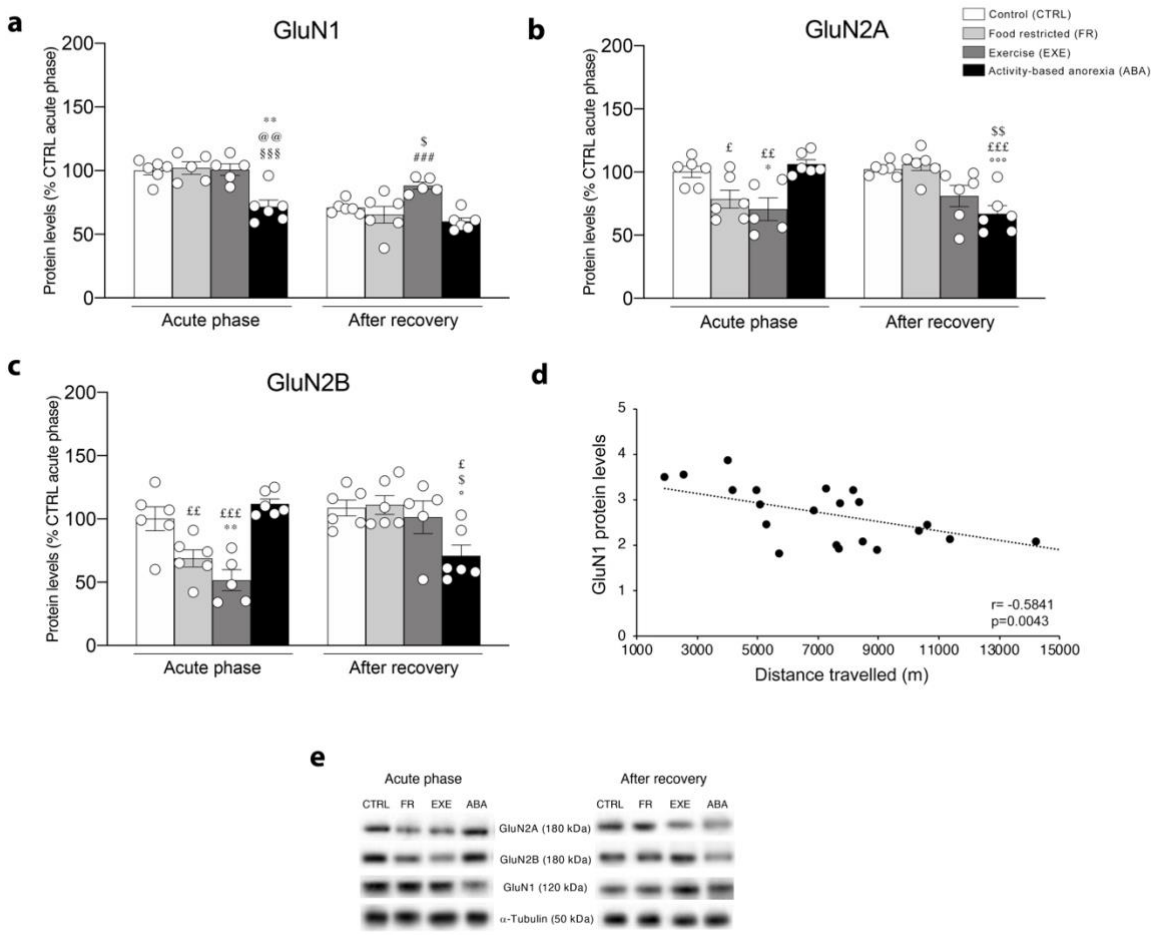


Figure 24. Effect of the ABA protocol on the NMDA receptor subunits expression in the post-synaptic density (PSD) of the mPFC in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of the obligatory subunit GluN1 (**a**) and the accessory subunits GluN2A (**b**) and GluN2B (**c**) of NMDA receptors are expressed as percentages of controls sacrificed at PND42 and represent the mean ± SEM of five-six rats per group. Panel (**e**) shows representative immunoblots for GluN1, GluN2A, GluN2B proteins in the PSD of mPFC.

Pearson's product–moment correlation (*r*) analyses between distance travelled at PND42 and GluN1 protein levels of ABA and EXE rats are shown in panel (**d**).

*p<0.05, **p<0.01 vs. CTRL-acute phase, $^{\$}$p<0.05, $^{\$\$}$p<0.01 vs. CTRL-recovery, §§§ p<0.001 vs. FR-acute phase, $^{@@}$ p<0.01 vs. EXE-acute phase, $^{£}$p<0.05, $^{££}$p<0.01, $^{£££}$p<0.001 vs ABA-acute phase, °p<0.05, °°°p<0.001 vs FR-recovery, $^{\#\#\#}$p<0.01 vs ABA-recovery (three-way ANOVA or two-way ANOVA followed by Tukey's multiple comparisons test). CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

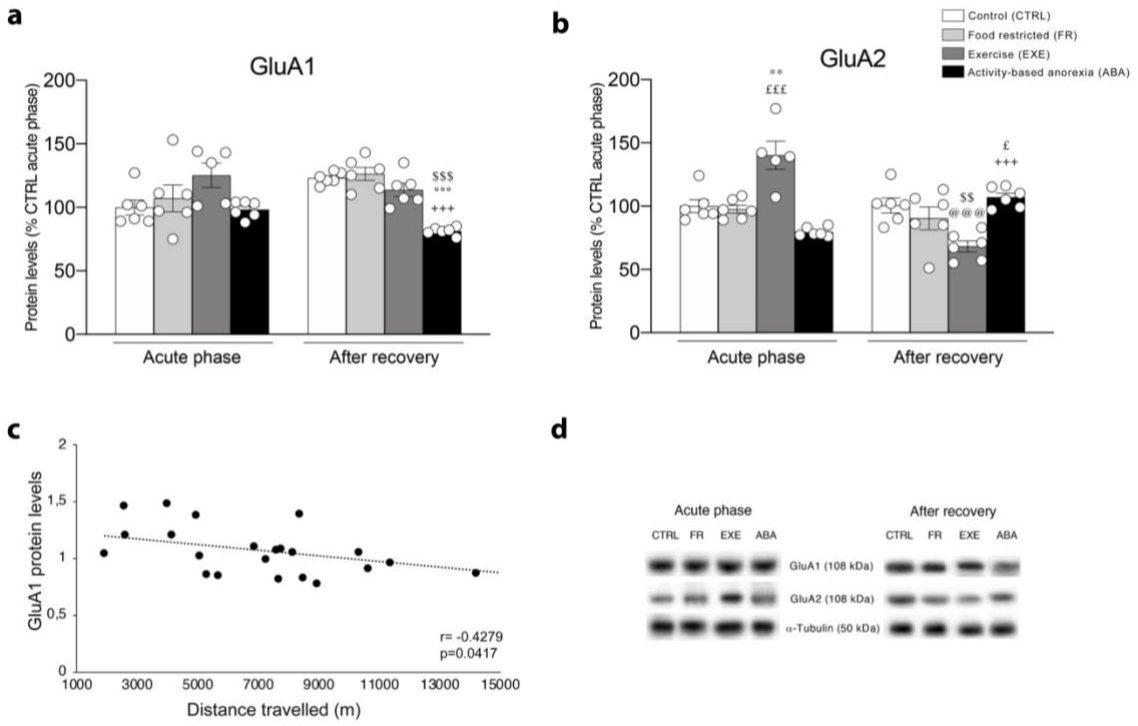


Figure 25. Effect of the ABA protocol on the AMPA receptor subunits expression in the post-synaptic density (PSD) of the mPFC in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of the GluA1 (**a**) and GluA2 (**b**) of AMPA receptors are expressed as percentages of controls sacrificed at PND42 and represent the mean ± SEM of five-six rats per group.
Panel (**d**) shows representative immunoblots for GluA1 and GluA2 proteins in the PSD of mPFC.
Pearson's product–moment correlation (*r*) analyses between distance travelled at PND42 and GluA1 protein levels of ABA and EXE rats are shown in panel (**c**).
**p<0.01 vs. CTRL-acute phase, $^{\$\$}$p<0.01, $^{\$\$\$}$p<0.001 vs. CTRL-recovery, $^{@@@}$ p<0.001 vs. EXE-acute phase, $^{£}$p<0.05, $^{£££}$p<0.001 vs ABA-acute phase, °°°p<0.001 vs FR-recovery, $^{+++}$p<0.01 vs EXE-recovery, $^{###}$p<0.01 vs ABA-recovery (three-way ANOVA or two-way ANOVA followed by Tukey's multiple comparisons test). CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

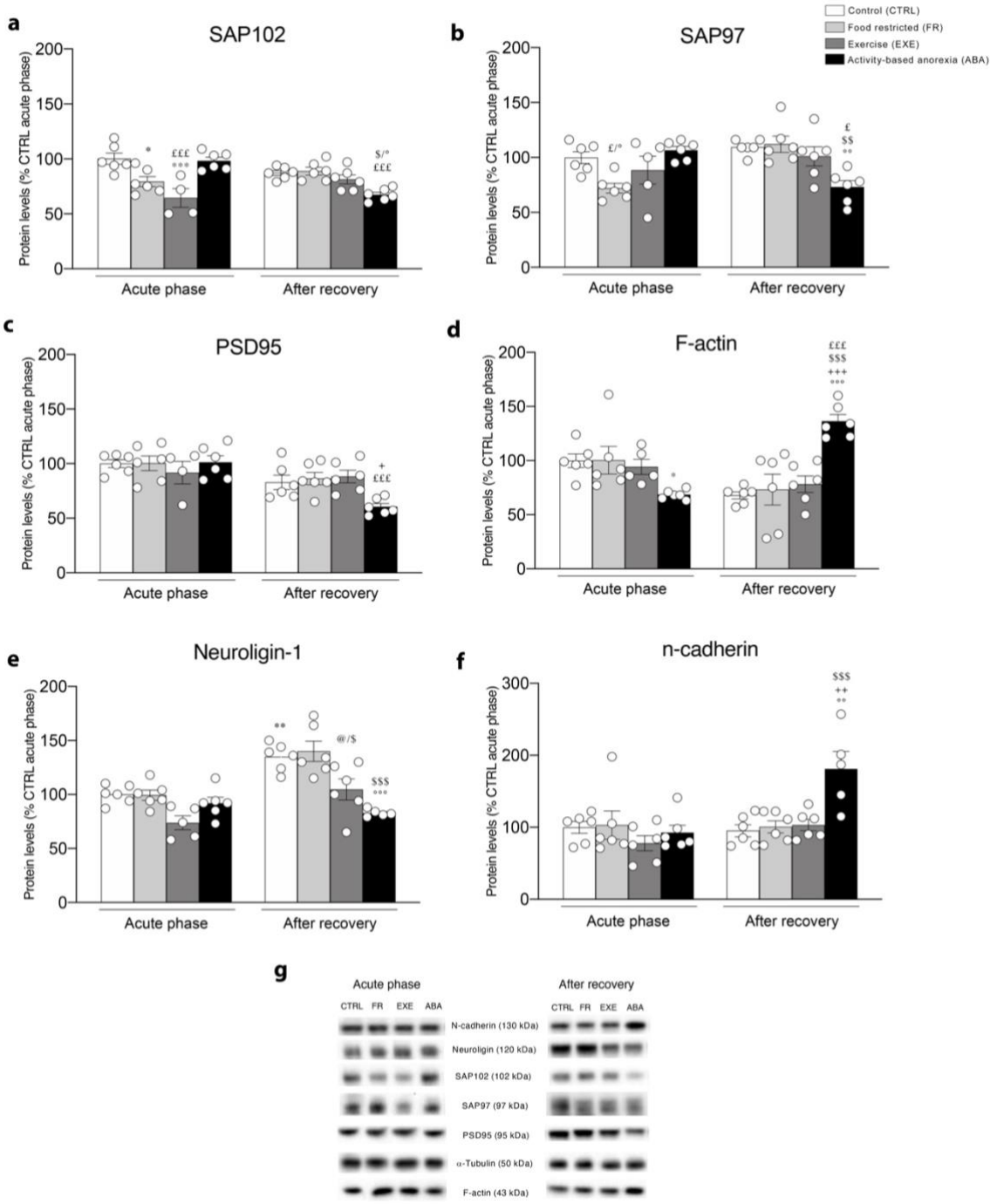


Figure 26. Effect of the ABA protocol on scaffolding proteins and on synaptic structural markers expression in the PSD of mPFC in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Protein levels of SAP102 (a), SAP97 (b), PSD95 (c), filamentous (F-)Actin (d), neuroligin-1 (NLGN1) (e) and N-cadherin (f) are expressed as percentages of controls sacrificed at PND42 and represent the mean ± SEM of five-six rats per group. Panel (g) shows representative immunoblots for SAP102, SAP97, PSD95, F-actin, NLGN1 and N-cadherin proteins in the PSD of mPFC. * $p<0.05$, ***$p<0.001$ vs. CTRL-acute phase, $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. CTRL-recovery, $^{\S}p<0.05$ vs. FR-acute phase, $^{@}p<0.05$ vs. EXE-acute phase, $^{£}p<0.05$, $^{£££}p<0.001$ vs ABA-acute phase, °$p<0.05$, °°$p<0.01$, °°°$p<0.001$ vs FR-recovery, $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ vs EXE-recovery (three-way ANOVA or two-way ANOVA followed by Tukey's multiple comparisons test). CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

a

Homogenate	Acute phase (PND 42)				After recovery (PND 49)				Three-way ANOVA (followed by Tuckey's post hoc test)
	CTRL	FR	EXE	ABA	CTRL	FR	EXE	ABA	Time x Exercise x Food restriction:
GluN1	100 ± 4	105 ± 3	125 ± 5 **/£££	96 ± 3	85 ± 3	100 ± 5	88 ± 3	97 ± 4	$F_{(1,39)}$=7.280, p=0.0102
GluN2B	100 ± 4	104 ± 8	135 ± 14 *	71 ± 2 §/@@@	61 ± 8	58 ± 5	78 ± 2	64 ± 3	$F_{(1,39)}$=9.666, p=0.0035
GluA1	100 ± 7	105 ± 7	140 ± 15 *	68 ± 3 §/@@@	113 ± 9	109 ± 6	128 ± 5	116 ± 6 £££	$F_{(1,39)}$=10.71, p=0.0023
GluA2	100 ± 12	96 ± 6	132 ± 12	83 ± 4 @@	97 ± 6	90 ± 7	90 ± 7	97 ± 5	$F_{(1,38)}$=7.755, p=0.0083
SAP97	100 ± 6	88 ± 8	130 ± 3	87 ± 6 @@	85 ± 5	80 ± 7	76 ± 4	83 ± 5	$F_{(1,39)}$=6.051, p=0.0186
Neuroligin-1	100 ± 8	105 ± 9	132 ± 5 *	97 ± 5 @	99 ± 5	101 ± 6	88 ± 5	103 ± 9	$F_{(1,39)}$=8.002, p=0.0073
F-actin	100 ± 4	109 ± 4	104 ± 5	74 ± 5 **/§§§/@@@	71 ± 2	79 ± 5	84 ± 4	79 ± 3	$F_{(1,39)}$=5.729, p=0.0216

b

Homogenate	Acute phase (PND 42)				Two-way ANOVA (followed by Tuckey's post hoc test)	After recovery (PND 49)				Two-way ANOVA (followed by Tuckey's post hoc test)
	CTRL	FR	EXE	ABA	Exercise x Food restriction:	CTRL	FR	EXE	ABA	Exercise x Food restriction:
GluN2A	100 ± 6	111 ± 7	145 ± 6***	112 ± 5@@	$F_{(1,19)}$=12.86, p=0.0020	104 ± 4	111 ± 6	123 ± 8	107 ± 2	$F_{(1,20)}$=4.549, p=0.0455
SAP102	100 ± 4	91 ± 2	120 ± 11	90 ± 3	$F_{(1,19)}$=3.691, p=0.0698	95 ± 6	94 ± 7	95 ± 3	100 ± 5	$F_{(1,20)}$=0.3692, p=0.5503
PSD95	100 ± 3	78 ± 4	100 ± 11	77 ± 4	$F_{(1,19)}$=0.01135 p=0.9163	103 ± 8	106 ± 8	97 ± 5	102 ± 6	$F_{(1,20)}$=0.05798, p=0.8122
n-cadherin	100 ± 3	103 ± 8	105 ± 5	82 ± 2@	$F_{(1,19)}$=6.653, p=0.0184	69 ± 5	79 ± 8	84 ± 9	100 ± 7	$F_{(1,20)}$=0.2045, p=0.6560

Table 7. Effects of FR, EXE and ABA induction in the acute phase of the pathology, at PND42, and after a 7-days recovery period (PND49) on the expression of critical determinants of the glutamate synapse, measured in the whole homogenate of the mPFC.

Protein expression data, expressed as % of CTRL-acute phase rats, represent the mean ± S.E.M. of at least 5 samples for each experimental group. Western blot data were analyzed by Three-way ANOVA (**a**) or Two-way ANOVA (**b**) followed by Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001 vs. CTRL-acute phase; § p<0.05, §§§ p<0.001 vs FR- acute phase; @ p<0.05, @@ p<0.01, @@@ p<0.001 vs EXE- acute phase; £££ p<0.001 vs ABA- acute phase. CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

4.2.1.2 Activity-based anorexia induction alters structural plasticity in the mPFC

To evaluate the impact of the anorexic phenotype on the structural remodeling of the mPFC, we examined cortical dendritic spine density and morphology in ABA animals both at the achievement of the anorexic phenotype (acute phase) and after a period of body weight recovery using a fluorescent dyolistic labeling technique. Since the molecular analysis presented in figures 2 and 3 show that only the combination between food restriction and hyperactivity specifically alters the cortical glutamate homeostasis, we decided to focus our attention on ABA versus CTRL group in the morphological study of the mPFC. To this end, another cohort of animals was randomly subdivided into four groups: 1) CTRL group, sacrificed at PND42; 2) ABA group, sacrificed at PND42; 3) CTRL group, sacrificed at PND49 and 4) ABA group, sacrificed at PND49.

The combination of food restriction and free wheel access reduced dendritic spine density in the acute phase, an effect that was restored after body weight recovery (Fig 27 a). While no ABA-induced changes were found in dendritic spine length (Fig 27 b), spine head size of ABA animals (Fig 27 c), in the acute phase of the disorder, are smaller than controls, while no changes were observed in recovered ABA animals.

Further, morphological analyses of dendritic spines were performed using a highly validated method of dendritic spines classification to evaluate the shape of all protrusions (mushroom, thin, stubby and filopodia, Fig 28 a). ABA induction reduced the percentages of mushroom-shaped spines, i.e. the mature spines where the synaptic communication takes place, in the acute phase of the disorder, whereas they were increased after the recovery of body weight (Fig 28 b). On the contrary, filopodia, i.e. the immature protrusions, were increased at PND42 in the mPFC of ABA rats, an effect that was restored at PND49 with body weight recovery (Fig 28 e). No changes were observed in the percentages of stubby- and thin-shaped spines (Fig 28 c, d). As shown in table 2, the ABA-induced reduction of head width size in the acute phase of the pathology appeared to be widespread as it affected mushroom-, thin- and stubby-shaped spines. Again, this reduction was restored at PND49 after body weight recovery. Two-way ANOVA of spine length, classified by their shape, revealed a significant interaction ABA procedure x time only for mushroom-shaped spines (table 8): ABA induction increased the length of mushroom-shaped spines at PND42, whereas body weight recovery restored their size (See Table 18 for statistics).

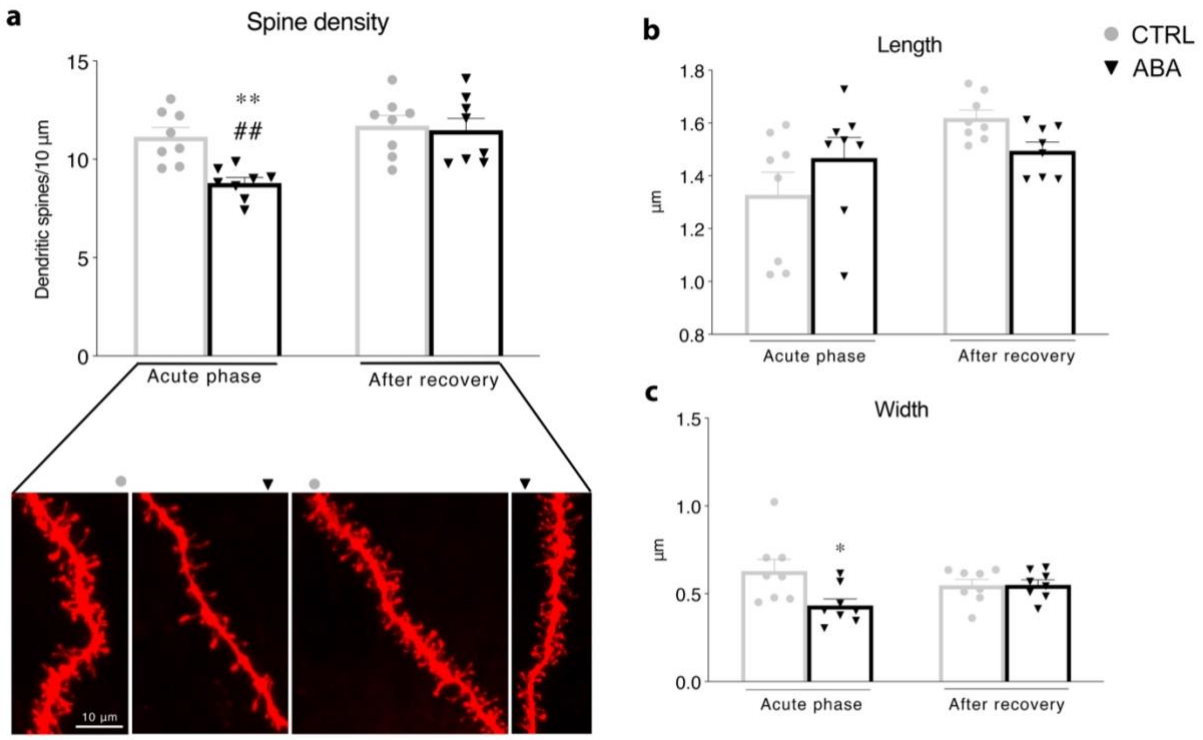


Figure 27. Effects of ABA induction on dendritic spine density and morphology in the mPFC measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Panel (**a**) shows total spine density and, below the graph, representative images of dendrite segments from the mPFC of CTRL and ABA animals evaluated at PND42 (left) and PND49 (right) are represented. Average spines length and average spines head width of the total spines measured are shown in panel (**b)** and (**c)**, respectively.
n > 3000 spines from at least 28 different neurons for each group, around 5 dendritic segments for each hemisphere, 8 hemispheres/group. Results are presented as the mean ± SEM. *p<0.05, **p< 0.01 vs. CTRL-acute phase; $^{\#\#}$p<0.01 vs ABA-recovery, (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL = control; ABA = activity-based anorexia.

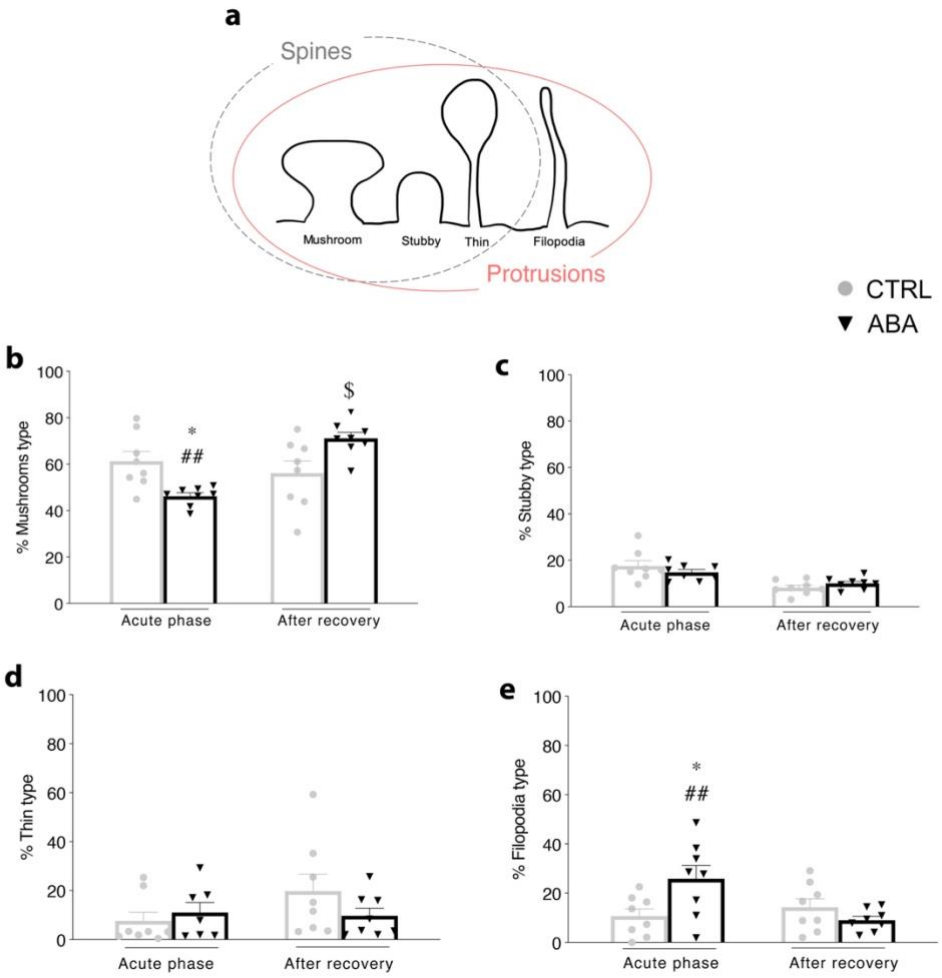


Figure 28. Effects of ABA induction on dendritic spine density and morphology in the mPFC measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Panel (**a**) shows a representative cartoon of different types of protrusions. The percentage of total protrusions belonging to different categories depending on their morphology is presented for mushroom- (**b**), stubby- (**c**), thin- (**d**) and filopodia (**e**)-shaped protrusions.
n > 3000 spines from at least 28 different neurons for each group, around 5 dendritic segments for each hemisphere, 8 hemispheres/group. Results are presented as the mean ± SEM. *p<0.05, **p< 0.01 vs. CTRL-acute phase; $^{\#\#}$p<0.01 vs ABA-recovery, $^{\$}$p<0.05 vs CTRL-recovery (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL = control; ABA = activity-based anorexia.

		Acute phase (PND 42)		After recovery (PND49)		Two-way ANOVA (followed by Tuckey's post hoc test)
		CTRL	ABA	CTRL	ABA	
Mushroom	length	1,4950 ± 0,1003	1,7475± 0,0404*	1,7176± 0,0265	1,2517± 0,0445	$F_{(1,28)}$=10.31, p=0.003
	width	0,7232± 0,0719	0,4862± 0,0435**	0,6222± 0,0336	0,4430± 0,0271	$F_{(1,27)}$= 5.734, p=0.0238
Stubby	length	0,6700± 0,0196	0,7119± 0,0236	0,7932± 0,0315	0,5320± 0,0166	$F_{(1,28)}$=3.830, p=0.0604
	width	0,4028± 0,0318	0,2831± 0,0184*	0,4342± 0,0280	0,2981± 0,0198$^{\#}$	$F_{(1,28)}$= 6.214, p=0.0189
Thin	length	1,3185± 0,0757	1,4016± 0,0788	1,4674± 0,0826	1,1081± 0,0799	$F_{(1,28)}$=1.644, p=0.2103
	width	0,5868± 0,0414	0,4012± 0,0477*	0,5648± 0,0467	0,4421± 0,0478	$F_{(1,28)}$= 5.060, p=0.0325

Table 8. Effects of ABA induction on the average spines length and head width, expressed as μm±SEM, of the mushroom-, stubby-, thin-shaped spines in the mPFC measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49).
*p<0.05, **p<0.01 vs. CTRL-acute phase; $^{\#}$p<0.05 vs ABA-recovery (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL = control; ABA = activity-based anorexia.

4.2.1.3 Activity-based anorexia induction alters the recency discrimination performance in the TOOR test

To evaluate whether temporal memory abilities in the ABA rat model may recapitulate that of anorexic patients, we exposed another cohort of CTRL and ABA rats, which reached the same behavioral phenotype presented in figures 14 and 23 (data not shown), to the temporal order object recognition test (TOOR). This cognitively-demanding test evaluates mPFC functionality through a recency recognition task, in which the animal's ability to differentiate between two familiar objects presented at different intervals is tested (Fig 29 a) (Barker & Warburton, 2011) (Barker et al., 2007; Manago et al., 2016).

Figure 29 panel d represents the performance during the test phase, calculated as recency discrimination index. While CTRL rats were spending more time exploring the object presented least recently, ABA rats in the acute phase failed to show any preference for the less recent object. Interestingly, despite the body weight recovery ameliorate recency discrimination in ABA rats at PND49, as compared to recency performance displayed at PND42, the temporal memory deficit persisted (See Table 19 for statistics).

Moreover, animals did not show any preference in exploring the different objects in sample phase 1 or sample phase 2 nor in the test phase, as shown in the total exploration time (Fig 29 b, c).

To evaluate causality between ABA phenotype and recency memory impairment, we run a Pearson correlation analyses between distance travelled during the last day of ABA induction and the body weight of ABA rats at both time points with the recency discrimination index. Interestingly, discrimination index correlated negatively with the distance travelled at PND42 (Fig 29 e) and positively with body weight of ABA rats (Fig 29 f).

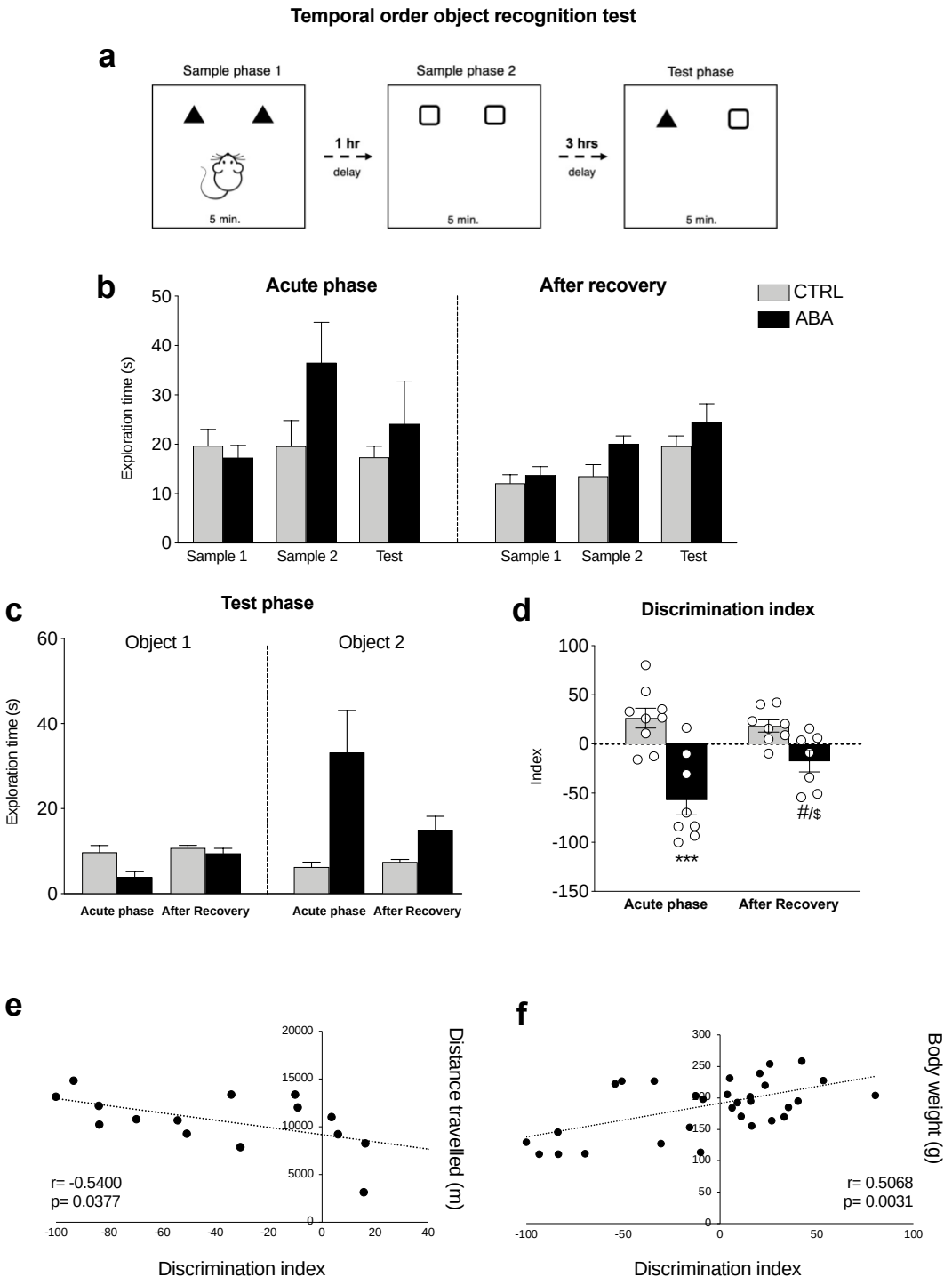


Figure 29. Schematic representation of the temporal order object recognition (TOOR) test (**a**), total exploration time among the three phases (**b**) exploration time of object 1 and object 2 measured in the test phase (**c**) discrimination index (**d**). Pearson's product–moment correlation (*r*) analyses between recency discrimination index and distance travelled at PND42 of ABA rats (**e**). Pearson's product–moment correlation (*r*) analyses between recency discrimination index and body weight (**f**) of ABA and CTRL rats at both PND42 and PND49. Recency discrimination index was calculated as (exploration time of object presented in sample phase 1 − exploration time of object presented in sample phase 2) / total time spent exploring both objects in the test phase. ***p<0.001 vs CTRL-acute phase; $p<0.05, vs. CTRL-recovery; #p<0.05 vs ABA- acute phase (two-way ANOVA followed by Fisher's LSD test). Histograms represent the mean ± SEM of at least seven rats per group. CTRL = control; ABA = activity-based anorexia.

4.2.2 **Discussion**

As discussed in paragraph 4.1.2, we found that adolescent ABA rats reduced food intake and progressively increased their physical activity leading to self-starvation and hyperactivity, further aggravating weight loss (Chowdhury et al., 2015; Routtenberg & Kuznesof, 1967). Of note, the maladaptive cycle of self-starvation and hyperactivity in ABA rats escalates rapidly and severely during adolescence, in agreement with the age-dependent risk for clinical AN described in the DSM-5 (DSM-V, 2013).

At molecular level, the reorganization of the glutamatergic synapse in the postsynaptic density of the mPFC was specifically driven by the combination of low caloric intake and hyperactivity, since both these conditions alone did not change the expression of the glutamate markers analyzed in the active area of the synapse. Interestingly, at PND42, physical activity per sé increased the translation of NMDA and AMPA receptor subunits; whereas hyperactivity induced by low caloric intake reduced their expression in the whole homogenate, suggesting an increased cortical vulnerability of ABA rats. At structural level, we demonstrated that the combination of weight loss and hyperactivity interfered with cortical dendritic spine formation, reducing their density, and influencing their maturation in the mPFC. In fact, the reduction of mushroom-shaped spines, which represent the most active type of dendritic spines (Bourne & Harris, 2007), and the increase of filopodia, i.e. the immature dendritic protrusions, may be indicative of an increased turnover of the immature dendritic structures reducing the communicative synapses and their strengthening and suggesting a mechanism to explain the cognitive deficits observed in anorexic patients (Olivo et al., 2019). In addition, the reduced spine density in the mPFC of ABA rats was coupled with a reduced head diameter, reflecting a shrinkage of the postsynaptic density area (Hering & Sheng, 2001), and with an impaired glutamatergic signaling via reduced expression and synaptic retention of specific glutamate receptors. Interestingly, such alterations are paralleled by reduced expression and altered synaptic localization of structural markers such as F-actin. Since synaptic function is positively associated with spine head volume, the PSD area and the number of glutamate receptors (Bosch & Hayashi, 2012), our data revealed that the observed molecular dysregulation of the glutamate synapse at the achievement of the anorexic phenotype correlates with ABA-induced hyperactive behavior, further corroborating the AN-induced weakening of cortical synaptic strength.

Of note, seven days of refeeding restored body weight and the observed structural alterations in the mPFC, even increasing the percentages of mushroom-shaped spine. Such effect may

reflect an adaptive response, or at least an attempt, of the mature synapses to restore a physiological synaptic communication. This is in line with the observation that, in humans, adolescent anorexic subjects display reduced grey matter volumes in the prefrontal cortex, decreased cortical thickness and subcortical volumes compared to healthy controls, an effect only partially retrieved upon refeeding (Frintrop et al., 2019; Kaufmann et al., 2020; Martin Monzon et al., 2017). Despite weight recovery canceled structural changes induced by the ABA phenotype, cytoskeletal instability in the mPFC of ABA rats is further confirmed at the molecular level via reduction of neuroligin-1 and PSD95, suggesting that these molecular alterations may account for functional persistent changes, as previously suggested in the mPFC of ABA rats even for the endocannabinoid system (Collu et al., 2019). Moreover, an overall reduction of the glutamatergic signaling in the postsynaptic density of the mPFC was still observed, indicating an AN-induced profound and persistent reorganization of the postsynaptic density composition. These data demonstrate an impaired synaptic localization of the main glutamate NMDA and AMPA receptor subunits since their expression was significantly reduced in the PSD fraction, but not in the whole cortical homogenate: accordingly, it appears that ABA induction has not influenced the translation process of these receptors but, rather, their synaptic retention. Since SAP102 and SAP97 in the postsynaptic density are essential for the synaptic localization of AMPA and NMDA receptors (Kim et al., 2005b; Vickers et al., 2006) and for anchoring such receptors to the postsynaptic membrane, the herein observed reduction indicates impaired delivery and reduced synaptic stability of glutamate receptors at the postsynaptic membrane.

Taken together, the complex set of structural and molecular analyses herein shown point to (mal)adaptive glutamatergic rearrangements occurring in the adolescent brain, that might contribute to alter the incentive motivational system and recency discrimination abilities to drive maladaptive behaviors in AN, as revealed by the significant correlations between glutamatergic receptors and recency discrimination index with ABA-induced hyperactive behavior and body weight loss. These events are functionally relevant since the long-term failure in recruiting critical glutamate determinants at synaptic level might reduce the transition to active dendritic spines, leading to a functional destabilization of the mPFC and conferring greater vulnerability to AN patients. This notion is reinforced by the evidence that the recency discrimination index in the temporal order object recognition test, which requires an intact and functional mPFC (Barker et al., 2007), was impaired. The dysregulated compartmentalization and reduced synaptic retention of critical glutamate determinants, as a result of the anorexic phenotype, indicate that, at the beginning of the TOOR test, the availability of NMDA and

AMPA receptors at active synaptic sites is reduced in the mPFC of ABA rats, a deficiency that may contribute to the memory impairment observed in both the animal model and AN patients (Amianto et al., 2013; Biezonski et al., 2016; Lamanna et al., 2019). In addition, weight recovery in ABA rats is not paralleled by memory recovery: in fact, a recency memory deficit is still present following the recovery period, underscoring a vulnerability trait for relapse. This possibility is corroborated by the evidence of a slower maturation of the prefrontal regulatory circuitry in AN patients, as shown by fMRI studies (Xu et al., 2017), in which AN adolescents exhibited altered maturation of the executive network, leading to impaired cognitive flexibility and working memory (Bohon et al., 2020; Fuglset, 2019; Olivo et al., 2019). Moreover, Milton and colleagues elegantly showed that body weight loss and reversal learning in ABA rats was prevented via chemogenetic suppression of the mPFC-nucleus accumbens shell pathway (Milton et al., 2020), suggesting that different cognitive domains might be involved in AN.

4.3 *Activity-based anorexia alters a periphery-to-brain neurometabolic axis: impact on hippocampal structural plasticity and spatial memory performance*

Manuscript in preparation

Physiological levels of exercise and appropriate dietary regimens are undoubtedly linked to improve quality of life, including brain health, function, and cognitive abilities. However, in a pathological condition such as AN, where physical activity is exaggerated and the dietary behavior is restricted, two positive factors became negative contributors for brain plasticity and connectivity via a malnutrition-induced impairment of metabolic flexibility (i.e. the ability to adapt to food restriction- induced mitochondrial stress and excitotoxicity). Among the molecules that might be involved in this process, the neurotrophin *Brain-derived Neurotrophic Factor* (BDNF) has been widely characterized as a positive mediator of the beneficial neuro-protective effects of the exercise, and it is well known for its role in brain development, including neuronal cell survival, dendritic arborization, synaptogenesis and plasticity (Kellner et al., 2014; Numakawa et al., 2018). Moreover, BDNF modulation is also critical for the rewarding mechanisms of drugs of abuse and for the modulation of cognition (Li & Wolf, 2015; Lu et al., 2014). In addition, as previously described (see paragraph 1.5) recent findings pointed out the critical involvement of BDNF in food intake and weight regulation.

In this scenario, a growing body of evidence supports the hypothesis that maladaptive behavior typical of AN might be sustained, at least in part, from a neurobiological point of view, by an altered crosstalk between peripheral signals and brain functions, via muscular outputs and appetitive modulators signals to the brain, arising as altered motivational behavior and cognitive performance. Therefore, our hypothesis relies on the possibility that food restriction, coupled with intense exercise, may activate a *muscle-to-brain* signal that via a PGC1α-FNDC5 mechanism promote the secretion of the myokine irisin, from myocytes in the blood circulation, that reaching the brain it can modulates BDNF expression in specific brain areas crucial for associative reward learning, such as the Hip.

To pursue this aim, we evaluated critical determinants of the neurometabolic axis focusing our attention on the *muscle-to-brain* crosstalk PGC1α/FNDC5/Irisin/BDNF pathway. We first investigated the metabolic state of muscle fibers, the role of peripheral signals coming from skeletal muscles, such as PGC1α and FNDC5 proteins and, then, their putative effects in the modulation of central effectors expression, i.e., BDNF, in the Hip. Moreover, to further elucidate the impact of ABA induction in regulating structural plasticity, we performed spine

density and morphology characterization of dendritic spines in the Hip of ABA rodents, either at the achievement of the acute phase of the disease and after a period of body weight recovery. Lastly, due to the tight connection between energy intake, dendritic spine morphology and cognitive performance (Mattson et al., 2018), to obtain a functional correlate of the molecular and structural alterations induced by the anorexic phenotype, we exposed ABA rodents to the spatial order object recognition (SOOR) test, a cognitive demanding test, based on the discrimination between a familiar or displaced position of an object, mainly driven by Hip (Barker et al., 2007).

4.3.1 **Results**

4.3.1.1 Activity-based anorexia hyperactivates skeletal muscle metabolism

Skeletal muscles are metabolic active tissue that react firstly to physical exercise, specifically when physical activity is excessive, and then to energy restriction, via regulation of metabolic processes to maintain their energy balance.

To investigate whether the anorexic phenotype may, via altered metabolic profile, negatively impact the periphery-to-brain crosstalk, we initially investigated *Glut4* (glucose transporter 4) and *Cpt1β* (carnitine palmitoyltransferase 1β) gene expression in soleus muscle. *Glut4* is an insulin-dependent glucose transporter generally associated with higher muscle oxidative capacity, which is responsible of the transport of glucose from the blood flow into the myocyte. *Cpt1β* is, instead, the skeletal muscle isoform of the mitochondrial enzyme that catalyzes the transfer of long chain fatty acids to carnitine across the mitochondrial inner membrane for subsequent β-oxidation.

Early after the induction of the anorexic phenotype, at PND42, *Glut4* expression is induced in both FR and ABA rodents (Fig 30 a), suggesting a main effect caused by the food restriction, whereas *Cpt1β* is reduced in EXE rats, while due to the combination of food restriction and exercise is increased (Fig 30 c). Interestingly, body weight recovery normalizes the levels of both *Glut4* and *Cpt1β* (Fig 30 b, d). No significant differences among groups were observed at PND49 (See Table 20 for statistics).

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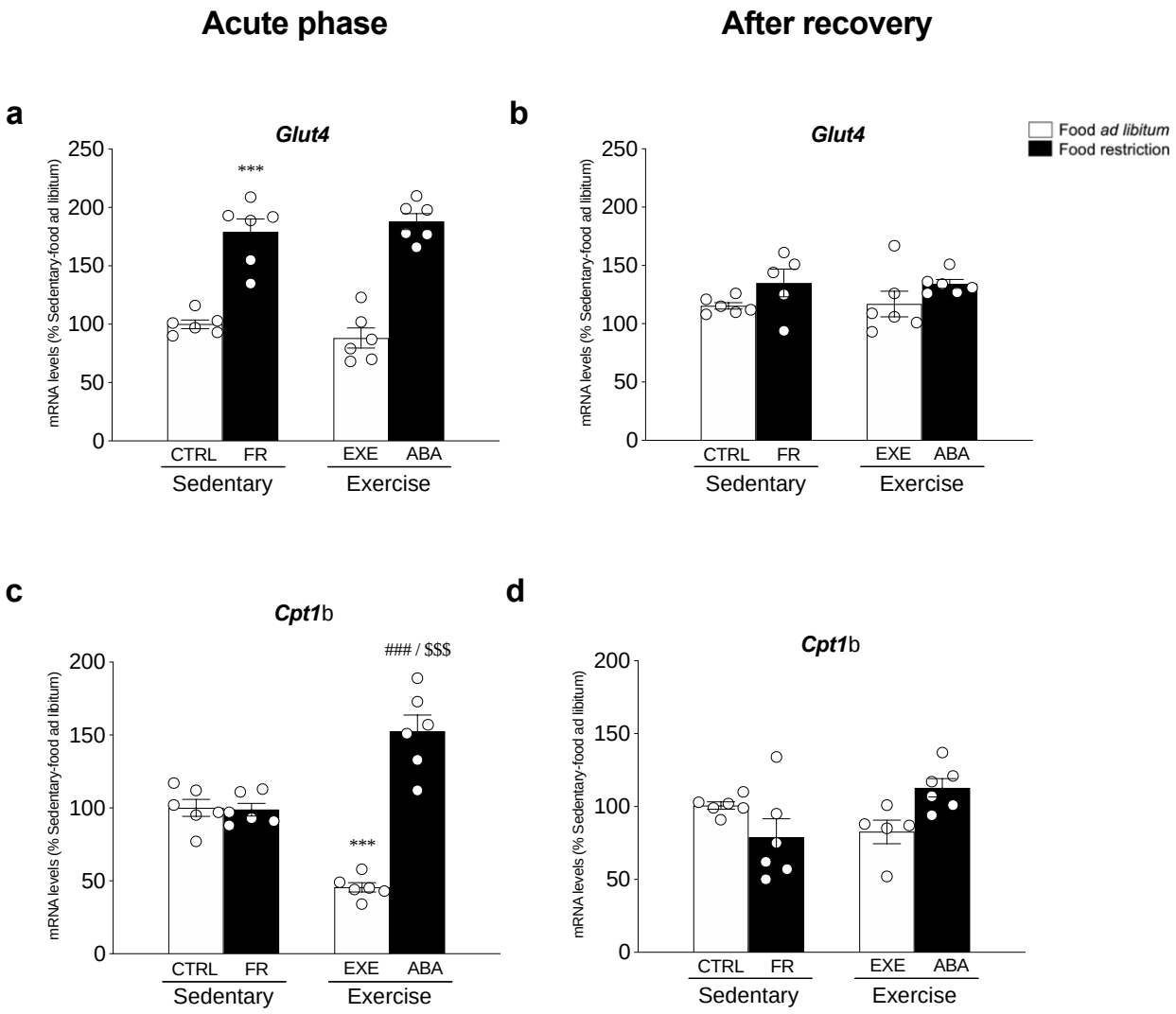

Figure 30. Effect of ABA induction on gene expression levels of *Glut4* and *Cpt1β* in the soleus muscle of adolescent female rats measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Panel (**a)** and (**c)** show *Glut4* and *Cpt1β* mRNA levels measured at PND42, panel (**b)** and (**d)** show *Glut4* and *Cpt1β* mRNA levels measured at PND49.

$^{***}p < 0.001$ vs. Food ad libitum-sedentary; $^{\$\$\$}p < 0.001$ vs. Food restriction-sedentary; $^{\#\#\#}p < 0.001$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test).

CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

4.3.1.2 Effect of the activity-based anorexia induction on the peripheral PGC1α/FNDC5/Irisin pathway

In the skeletal muscle, PGC1α has a central role in mediating many of the metabolic effects of exercise, such as the mitochondrial biogenesis, and in promoting the metabolic switch of muscle fibers from glycolytic to oxidative metabolism. It is well-known that physical exercise increases PGC1α expression in the muscle, that in turn stimulates the transcription and localization of a PGC1α-dependent transmembrane protein: FNDC5, an essential regulator of metabolic homeostasis, and precursor of the myokine named Irisin.

In our experimental condition, physical exercise induces the overexpression of PGC1α in the soleus muscle independently from dietary regimen, in fact both EXE and ABA groups show an increase of PGC1α in the acute phase of the pathology (Fig 31 a), an effect that is still present after body weight recovery only in EXE group (Fig 31 b). In ABA rodents, PGC1α protein levels are normalized (Fig 31 b). Interestingly, differently from PGC1α, the expression levels of FNDC5 are induced only in ABA rodents (Fig 31 c), suggesting a hyperactivation of the axis specifically resulting from the combination of food restriction and physical exercise. After body weight recovery, FNDC5 protein levels show a tendency toward reduction, although without statistical significance (Fig 31 d).

Next, we measured the cleavage product of FNDC5, irisin, in the plasma. Similar to FNDC5 expression in the soleus of ABA rats, plasmatic levels of irisin tend to increase at PND42 whereas are slightly reduced at PND49 in ABA rats, despite the body weight is restored (Fig 32) (See Table 21 for statistics).

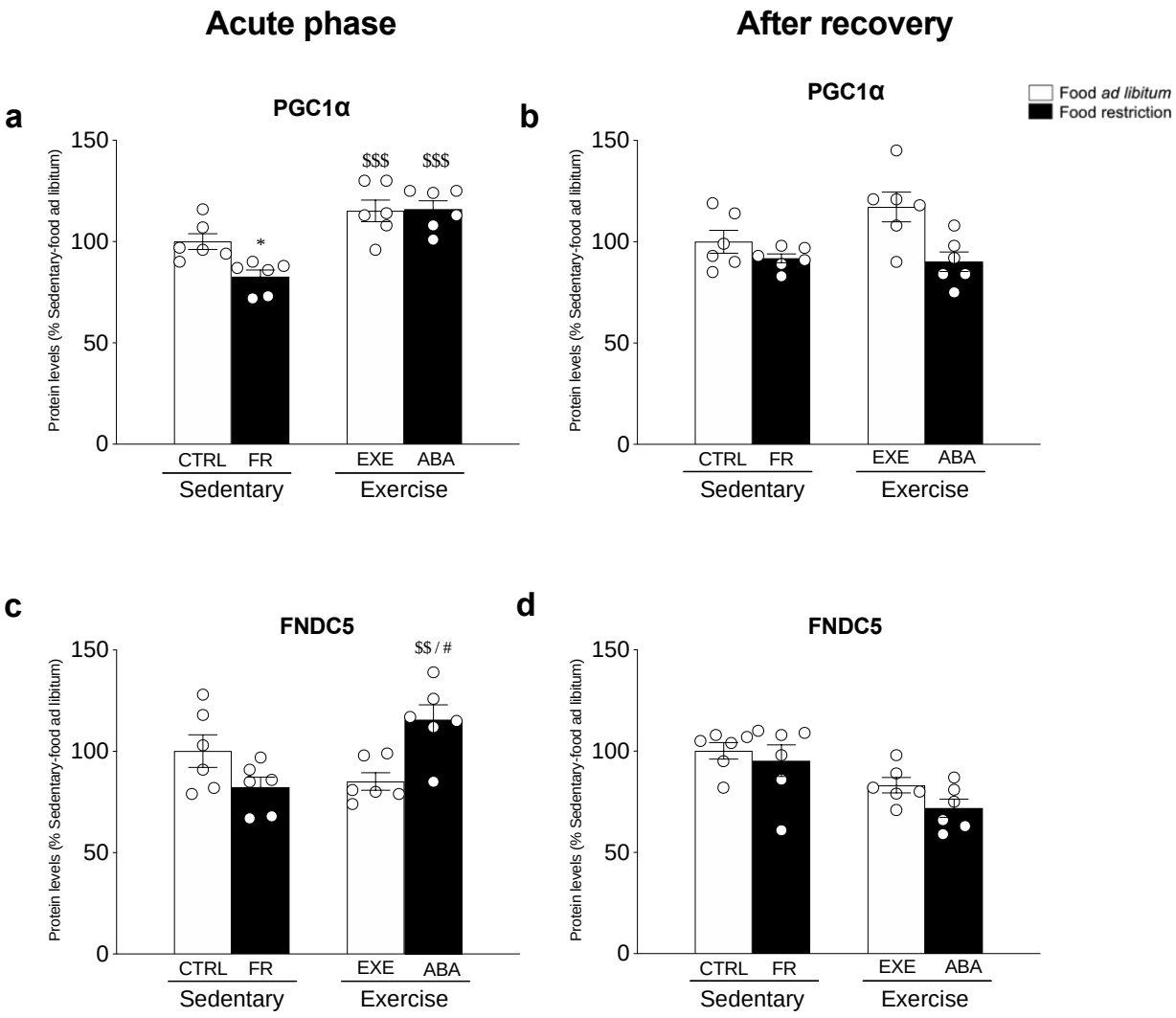

Figure 31. Effect of ABA induction on protein expression levels of PGC1α and FNDC5 in the homogenate of soleus muscle collected from adolescent female rats in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). Panel (**a)** and (**c)** show PGC1α and FNDC5 protein levels measured at PND42, panel (**b)** and (**d)** show PGC1α and FNDC5 protein levels measured at PND49.

$^{*}p < 0.05$ vs. Food ad libitum-sedentary; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. Food restriction-sedentary; $^{\#}p < 0.05$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test).

CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

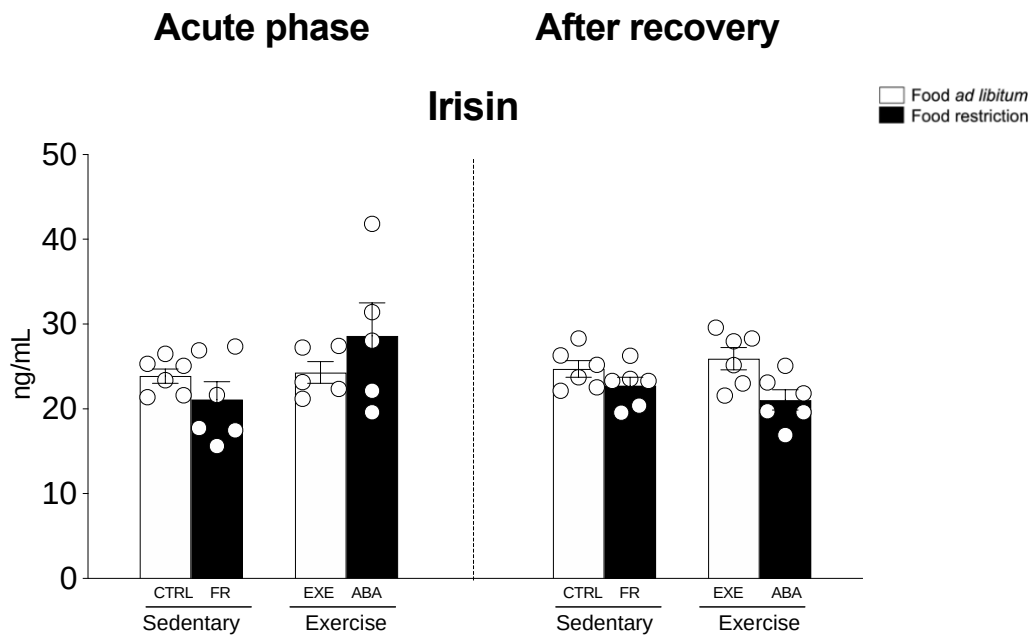

Figure 32. Effect of ABA induction on plasma levels of Irisin measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49).
CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

4.3.1.3 Effect of the activity-based anorexia induction on BDNF expression in hippocampus

In line with the hypothesis that Irisin is able to modulate BDNF in hippocampal neurons via PGC1α/FNDC5 mechanism, we next measured BDNF expression levels both in terms of transcription and translation in the hippocampus.

Accordingly with the literature, physical activity induced the overexpression of the *Bdnf* gene at PND42 independently from food restriction, in fact this effect was observed in both EXE and ABA groups (Fig 33 a). Conversely, after 7-days of recovery *Bdnf* gene expression in the ABA group is significantly reduced (Fig 33 b), whereas no changes were observed in EXE and FR groups. The mature form of BDNF (mBDNF) recapitulate the effect on gene transcription showing a significant increase of mBDNF protein levels in respect to CTRL only in the EXE group at PND42 (Fig 33 c), as expected. Of note, the combination of food restriction and exercise induced a dichotomy between transcription and translation: in fact *Bdnf* gene expression is increased whereas mBDNF protein levels is significantly reduced (Fig 33 d), suggesting an ABA-induced impairment in BDNF-dependent neuroplastic mechanisms. At PND49 the food restriction condition, independently from the physical exercise, induced a reduction in mBDNF (Fig 33 d).

To further dissect the effect of the AN phenotype on the BDNF system in the hippocampus, we measured the protein expression levels of its high-affinity receptor TrkB, both in terms of phosphorylation and total protein levels. According to mBDNF, in the acute phase of the pathology the combination of food restriction and physical exercise reduced the phosphorylation levels of TrkB in y706 and the expression levels of TrkB (Fig 34 a), suggesting an overall reduction of the system induced by the ABA condition. This effect persists despite body weight recovery (Fig 34. b). No changes were observed by the single conditions, food restriction or exercise alone, at both time points (Fig 34 a, b) (See Table 22-23 for statistics).

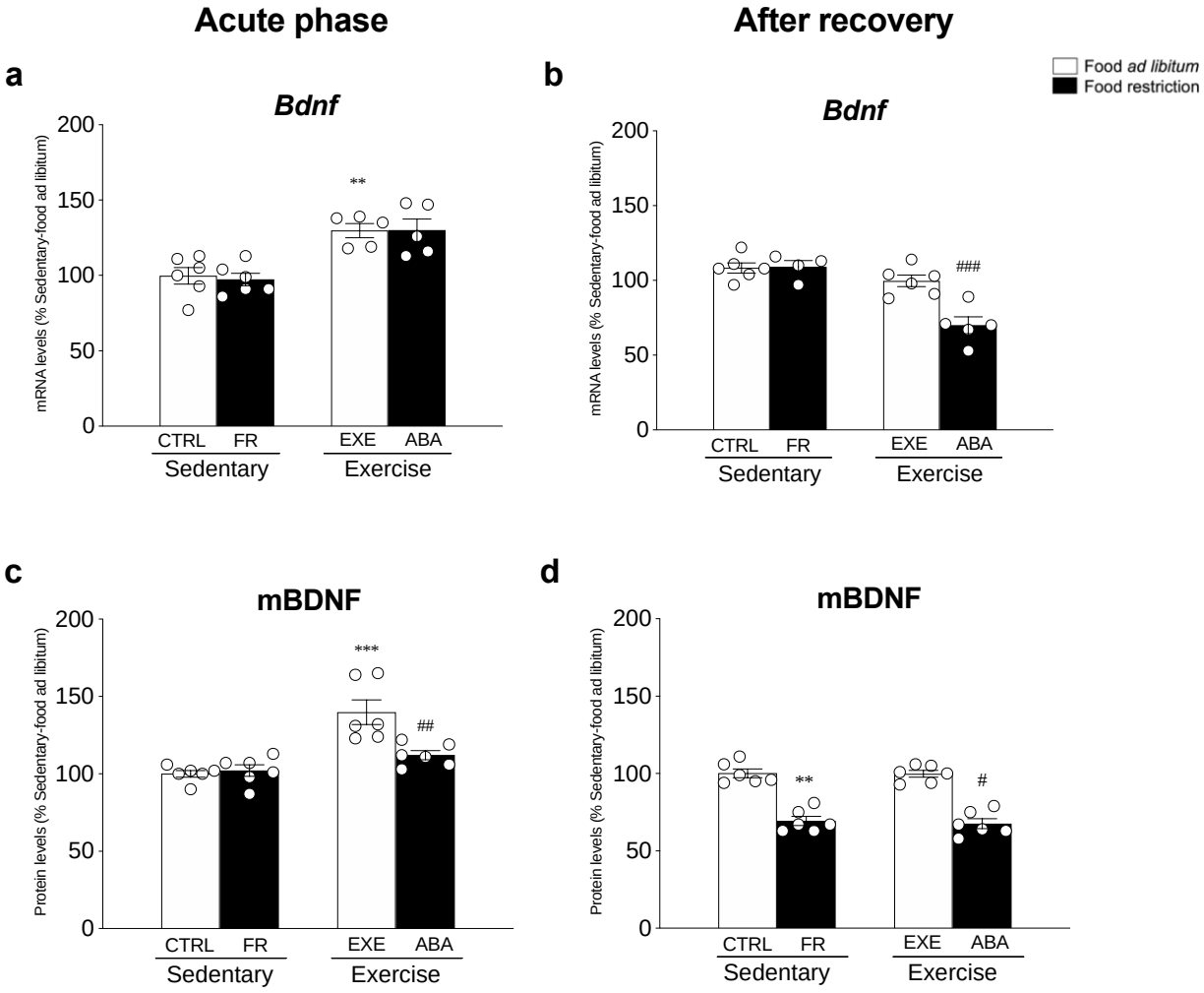

Figure 33. Effect of ABA induction on *Bdnf* gene expression and mBDNF protein levels in the Hippocampus (Hip) of adolescent female rats in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). *Bdnf* gene expression levels are shown in panel (**a)** at PND42 and in panel (**b)** at PND49. mBDNF protein expression levels, measured in the hippocampal crude membrane fraction, are shown in panel (**c)** at PND42 and in panel (**d)** at PND49**.**

$^{**}p < 0.05$, $^{***}p < 0.001$ vs. Food ad libitum-sedentary; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test).

CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

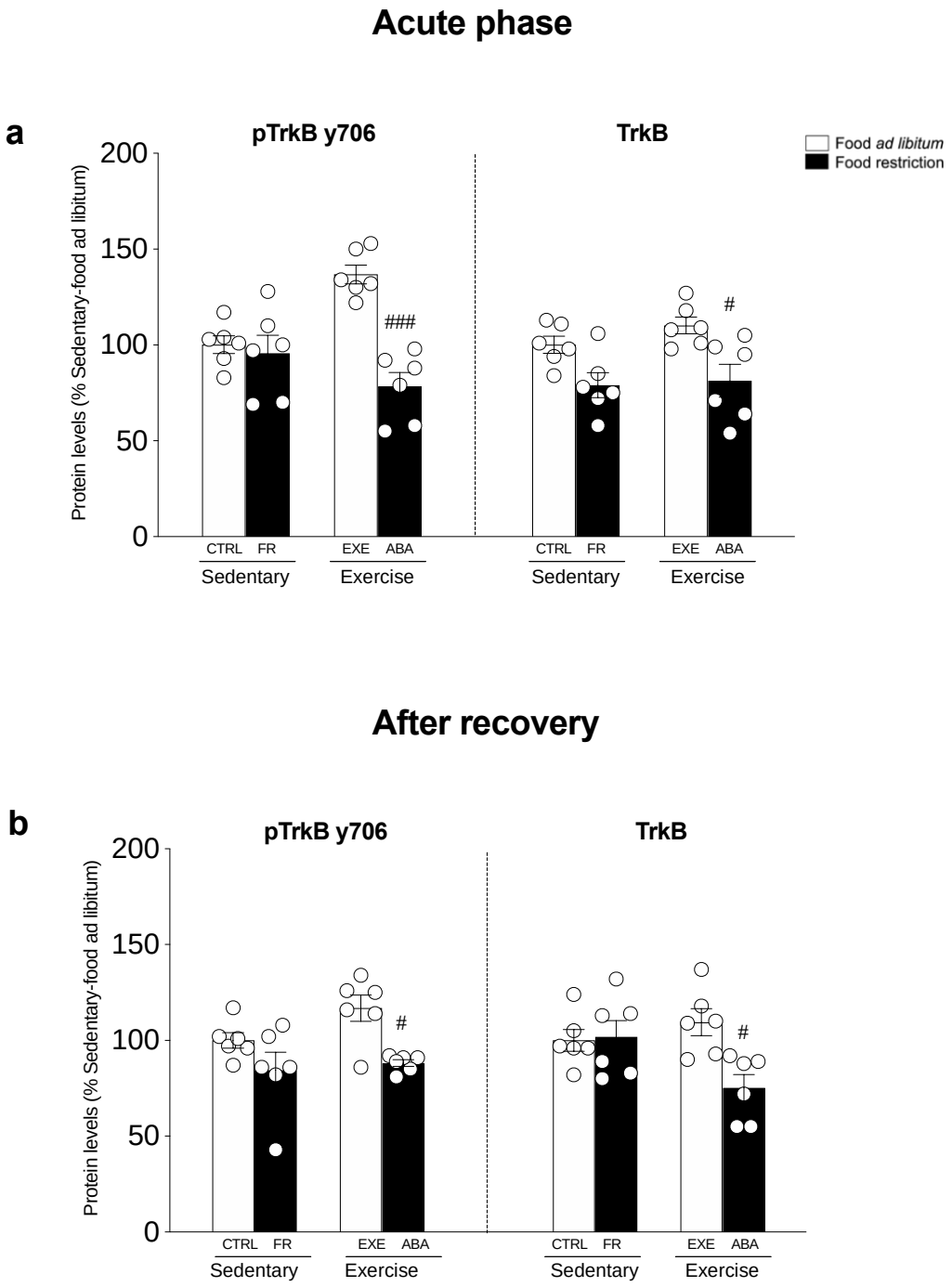

Figure 34. Effect of ABA induction on phosphorylation and protein expression levels of TrkB receptor in the crude membrane fraction of Hip of adolescent female rats in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). TrkB protein levels measured at PND42 are shown in panel (**a)** and measured at PND49 in panel (**b).**

$^{\#}p < 0.05$, $^{\#\#\#}p < 0.05$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test).

CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

4.3.1.4 Activity-based anorexia induces long lasting changes in synaptic stability

The postsynaptic density protein 95 (PSD95) is recruited and localized in the post-synaptic density region of synapses, thus contributing to synaptic strength and structure. Moreover, PSD95 is an index of post-synaptic density integrity and an important regulator of dendritic spines in vivo, thus giving a critical contribution in synaptic functionality (Berry & Nedivi, 2017; Broadhead et al., 2016).

In our experimental condition, the induction of the ABA phenotype induced a significant reduction of PSD95 protein levels in the hippocampal crude membrane fraction, whereas its levels are not affected by the food restriction or physical exercise per sé (Fig 35 a). Interestingly, even after the period of body weight recovery, PSD95 protein levels specifically in the ABA group remain reduced (Fig 35 a), suggesting that only the combination of food restriction and hyperactivity is able to alter the structure and morphology of dendritic spine in the hippocampus (See Table 23 for statistics).

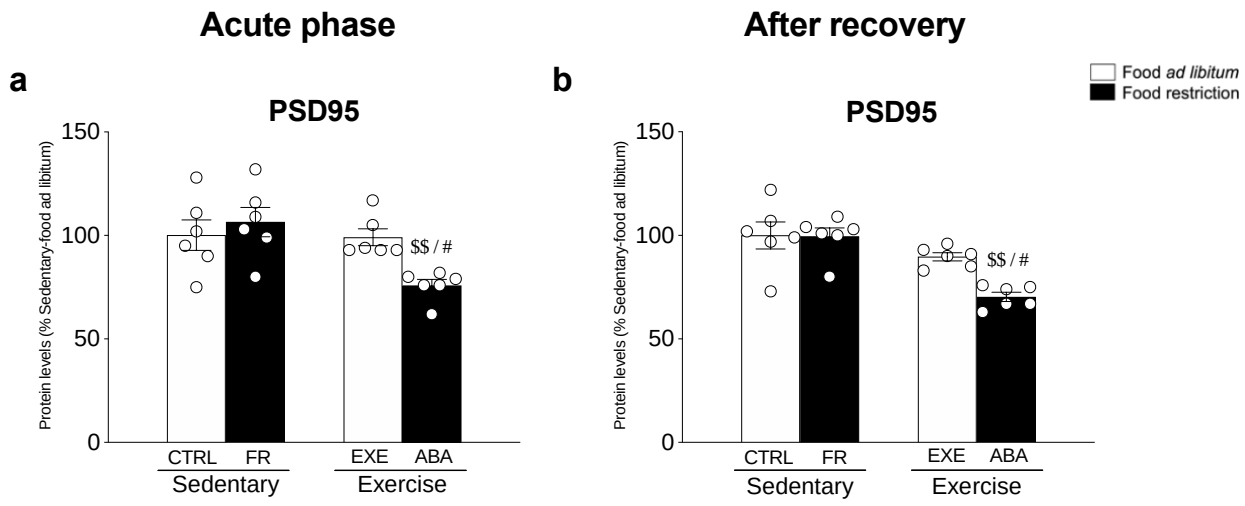

Figure 35. Effect of ABA induction on protein expression levels of PSD95 measured in the hippocampal crude membrane fraction of adolescent female rats in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49). PSD95 protein levels measured at PND42 are shown in panel (**a)** and measured at PND49 in panel (**b).**

$^{\$\$}p < 0.01$, vs. Food restriction-sedentary; $^{\#}p < 0.05$ vs. Food ad libitum-exercise (two-way ANOVA followed by Tukey's multiple comparisons test).

CTRL = control; FR = food-restricted; EXE = exercise; ABA = activity-based anorexia.

4.3.1.5 Activity-based anorexia alters structural plasticity in the hippocampus

Experience-dependent changes in spine density and morphology are associated with modifications in synaptic strength and neurotransmission; it has also been shown that these mechanisms characterize the most general form of synaptic plasticity behind memory formation. To understand whether the ABA-induced dysregulation of PSD95 levels, that is an index of post-synaptic density integrity, is paralleled by an altered spine structure, that, in turns, likely alter memory functions and might underpin the motivational mechanisms that fuel anorexic behaviors, we performed spine density and morphology analysis using a dyolistic labeling technique. We focused our attention on the Hip of only CTRL and ABA adolescent rats, early after the induction of the anorexic phenotype and after a period of body weight recovery.

According to PSD95 levels, the combination of food restriction and hyperactivity caused a significant reduction in spine density in the acute phase of the pathology (Fig 36 a). Interestingly, this reduction in spine density is paralleled by a reduction in the percentages of mushroom-shaped spines, the mature and active spines (Fig 36 c), and by an increased percentage of immature thin-shaped spines (Fig 36 d). Even though at PND49, after a recovery period, spine density is restored (Fig 36 a), a reduced percentages of active mushroom-shaped spines and an increased percentages of thin-shaped spines is still present (Fig 36 c, d), suggesting a persistent alteration in the hippocampal morphology of ABA rats. Of note, at PND49, head width of mushroom-shaped spines is increased (Fig 36 e), thus increasing their active surface, reflecting a protective response or an attempt of the mature synapses, which are reduced in number, to restore a physiological synaptic communication (See Table 24 for statistics).

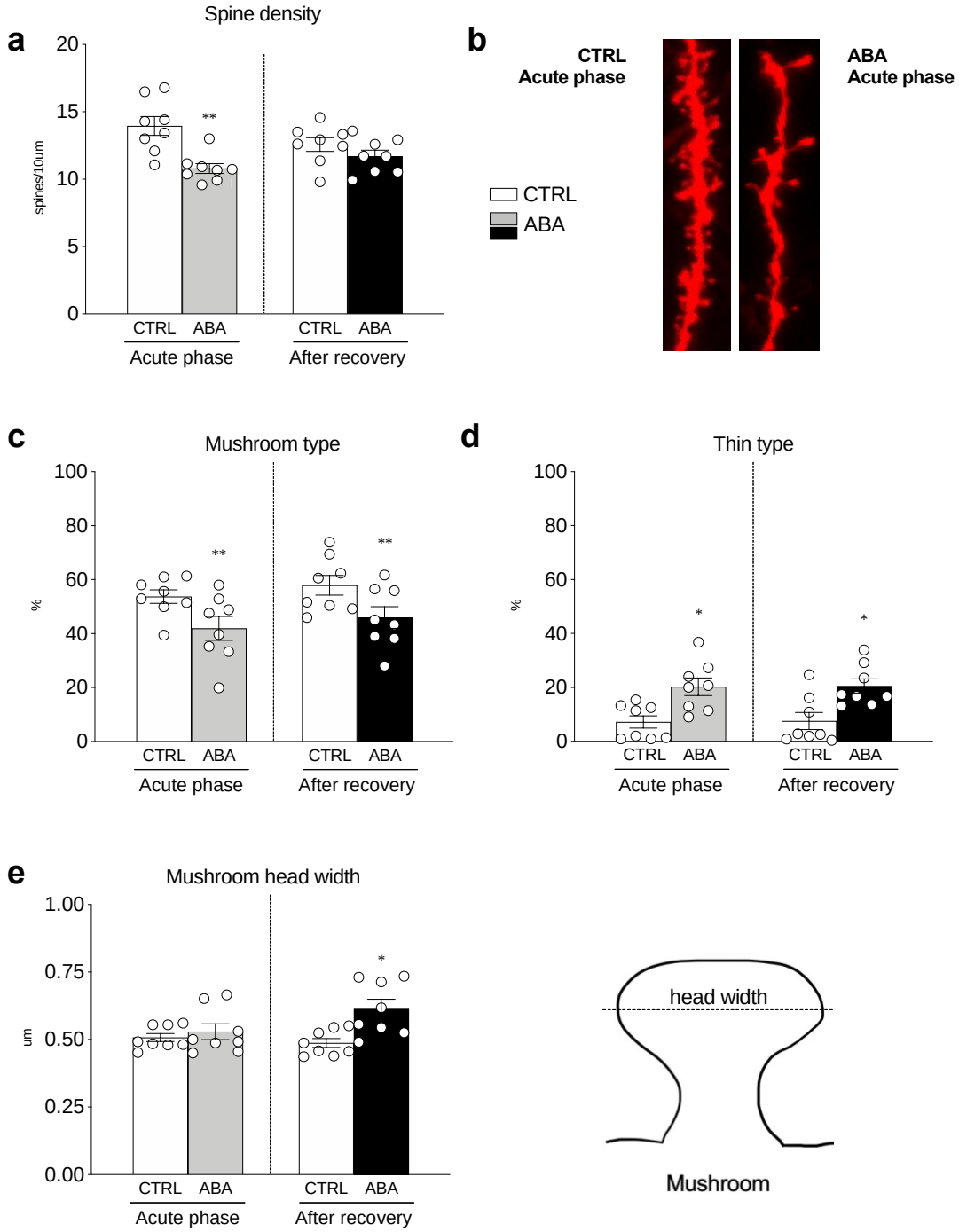

Figure 36 Effects of ABA induction on dendritic spine density and morphology measured in the acute phase of the pathology (PND42) and after a 7-days recovery period (PND49) in Hip. Panel (**a**) shows total spine density and panel (**b**) representative images of dendrite segments from CTRL and ABA animals evaluated at PND42. Percentage of mushroom- and thin-shaped spines are shown in panel (**c)** and (**d)**, respectively. Panel (**e)** show mushroom spines head width (left), and a schematic representation of a mushroom-shaped spine (right). n > 2000 spines from at least 25 different neurons for each group, around 5 dendritic segments for each hemisphere, 8 hemispheres/group. Results are presented as the mean ± SEM.
$^{*}p<0.05$, $^{**}p< 0.01$ vs. CTRL-acute/recovery, (two-way ANOVA followed by Tukey's multiple comparisons test).
CTRL = control; ABA = activity-based anorexia.

4.3.1.6 Activity-based anorexia impacts the spatial memory object recognition of rodents in the SOOR test

To assess whether molecular changes and the structural alterations, observed in the hippocampus of ABA animals, might reflect a functional deficit, we exposed another cohort of CTRL and ABA rats (Experiment 4) to the Spatial Order object Recognition (SOOR) Test (Fig 37 a). During the SOOR test, we measured the exploration time (s) for each animal for either the displaced or the not displaced object, both in the training phase and in the test phase, dissecting the specific contribution of each object. SOOR test were performed in the acute phase of the pathology and after body weight recovery.

During the training phase no differences were observed in the exploration time of the left and the right objects within the two groups (Fig 37 b), at both time points, a result that allowed us to establish that all the animals had no preference for the right- and the left-placed objects. Of note, during the test phase, CTRL animals reduced the exploration time of the object not-displaced, at both time point (Fig 37 c), meaning that CTRL rats were more interested in exploring the object that was displaced from the original position, as expected. Interestingly, early after the induction of the ABA phenotype, ABA rats showed no differences in the exploration time between the displaced and not-displaced objects (Fig 37 d), revealing that the combination of food restriction and hyperactivity alters spatial recognition memory preventing animals to distinguish between the position of the two objects. Weight recovery was not capable to restore ABA rodents' ability to recognize the displaced object, in fact, even after recovery no differences in the exploration time between the displaced and not-displaced object were detected (Fig 37 e).

The discrimination index showed a significant impairment in the spatial recognition memory of ABA rodents in both the acute phase of the disease and after the period of body weight recovery, highlighting the strong and persistent impact of the ABA condition on spatial memory (Fig 37 f) (See Table 25 for statistics).

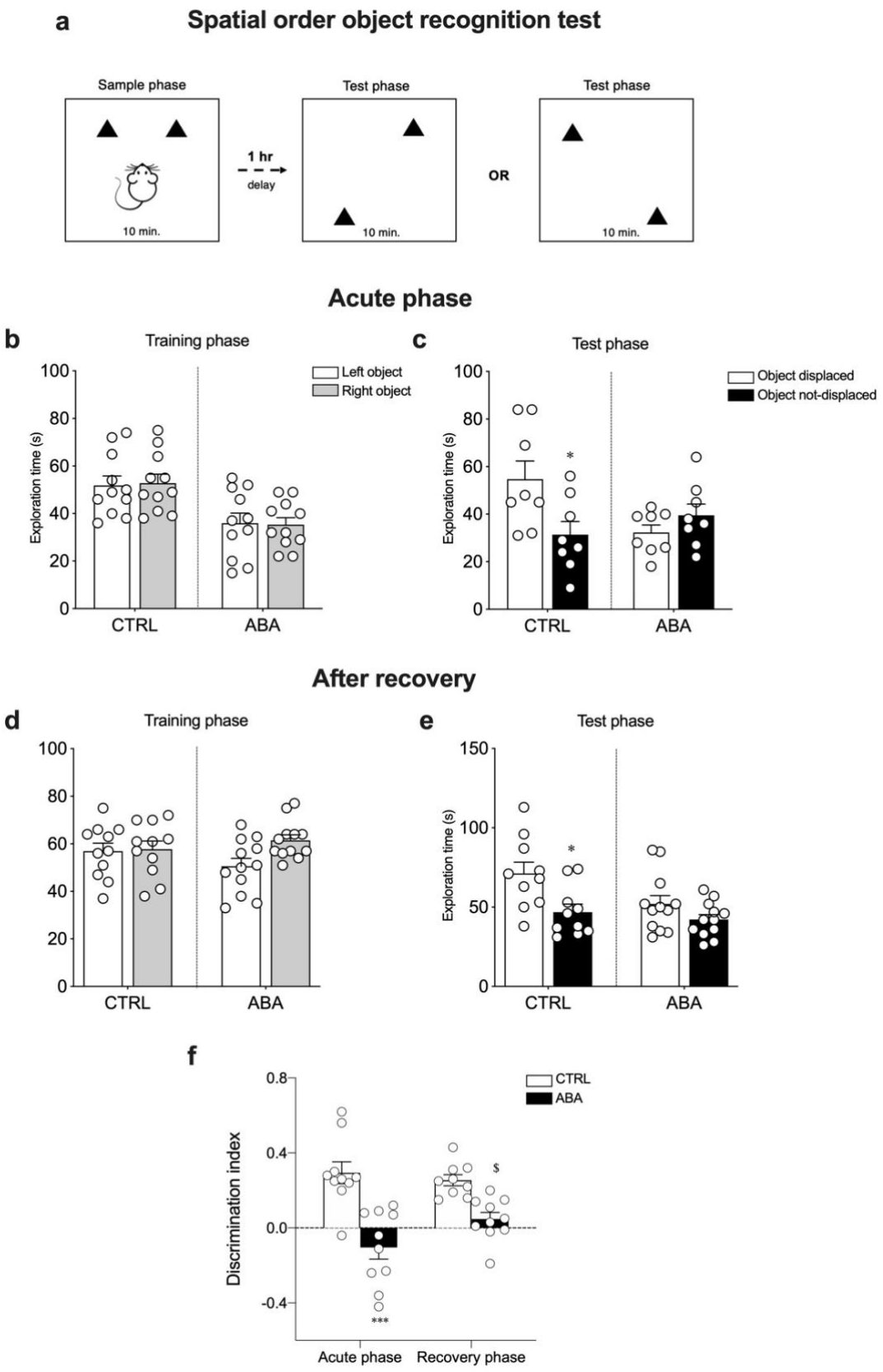

Figure 37. Exploration time (sec) measured during the training (**a, c**) and the test (**b, d**) phase of the spatial order object recognition (SOOR) test performed by CTRL and ABA groups in the acute phase and after recovery, respectively. Recency discrimination index, calculated as (exploration time of object not displaced − exploration time of object displaced) / total time spent exploring both objects in the test phase, is presented in panel (**e**). ***p<0.001 vs CTRL-acute phase; $^{\$}$p<0.05, vs. CTRL-recovery (two-way ANOVA followed by Tukey's multiple comparisons test). CTRL = control; ABA = activity-based anorexia.

4.3.2 **Discussion**

In attempt to further elucidate the neurobiological underpinnings of AN we focused our attention on the hypothesis that peripheral signals might modulate central responses, evaluating a *periphery-to-brain* crosstalk, that might be critical in the generation of aberrant outcomes typical of the disease.

First, we investigated whether the anorexic phenotype impacts the periphery-to-brain crosstalk via alterations of the metabolic profile in skeletal muscles. The skeletal muscle is one of the most dynamic and plastic tissues of the whole organism, in fact it is able to rapidly modulate, in response to exercise, the value of energy production, blood flow and the use of substrates (Frontera & Ochala, 2015; Gaitanos et al., 1993). Being rich in mitochondria, the muscle can produce energy through the oxidative phosphorylation mechanism. The energy metabolism of the muscle depends on the use of glucose, fatty acids and amino acids resulting from protein degradation (Westerblad et al., 2010). Physical exercise can be aerobic, anaerobic, intense, and short or long-lasting, and depending on the type of exercise the substrates that are metabolized to produce energy are different. In this scenario, the skeletal muscle is unique in its ability to rapidly increase its rate of energy consumption in situations where explosive contractions are required. To evaluate the muscle metabolic status, we measured the gene expression levels of some metabolic markers that are indicative of the consumption of glucose and fatty acids, such as *Glut4*, the insulin-dependent glucose transporter, and *Cpt1β,* a mitochondrial enzyme involved in β-oxidation, which is required for fatty acids to enter the mitochondrial matrix and be processed (Lundsgaard et al., 2018).

The overexpression of *Glut4* and *Cpt1β* that we observed early after the induction of the anorexic phenotype, suggests that the combination of caloric restriction and intense physical exercise alters the muscle cell metabolism, boosting the cell glucose uptake and the fatty acid oxidation (FAO). In the acute phase of the disease, the levels of gene expression of the glucose transporter *Glut4* were increased in both ABA and FR groups compared to the CTRL and EXE animals. This effect was related to caloric restriction per sé and might be considered as an adaptive response set in motion by muscle tissues to try to compensate for an ever-decreasing availability of circulating glucose. In fact, following the recovery of body weight, with glucose intake restoration, *Glut4* levels were restored to control levels. It is interesting to note that *Cpt1β* gene, as a key regulator of mitochondrial FAO in skeletal muscle, is instead induced only when intense exercise was combined with reduced caloric intake, thus, highlighting a

condition of potential energy deficit that required the activation of the β-oxidation as a backup source of energy substrates. On the other hand, reduced RNA levels of *Cpt1β* in EXE animals suggest that following a moderate physical exercise, counterbalanced by an appropriate caloric intake, the muscle is able to use other sources than degrading fatty acids. Moreover, after body weight recovery, *Cpt1β* gene expression levels are restored, reinforcing the hypothesis that *Cpt1β* induction is required only in the case of the combination of intense physical exercise and food restriction.

The overexpression of PGC1α, a transcriptional co-activator that regulates mitochondrial biogenesis and respiratory function, in skeletal muscles is implicated in muscle fiber-type switching. It has been shown that skeletal muscles from PGC1α transgenic mice and pigs, in which PGC1α gene is over-expressed under the porcine MCK (muscle creatinine kinase) promoter (vector MCK-PGC1α), have increased expression levels of oxidative fiber markers, and decreased expressions of glycolytic fiber genes (Zhang et al., 2017), further pointing to fiber-type conversion. Thus, the ABA-induced overexpression of PGC1α in the acute phase of the disease might promote a metabolic switch of muscle fibers, from fast-glycolytic fibers to slow-oxidative fibers, resulting in qualitative changes from glycolytic to oxidative energy generation. This increase of the fast-to-slow fiber-type conversion might play a crucial role in the long-lasting maintenance of the anorexic phenotype because it improves endurance performance and resistance to fatigue despite the low energy intake.

Of note, skeletal muscle plays crucial roles not only in regulating the energy balance and in different metabolic processes, but it is also important as a secretory organ to produce exercise-induced hormone-like myokines, such as irisin. Irisin is produced via PGC1α/FNDC5 mechanisms following physical exercise, through the proteolytic cleavage of the N-terminal part of FNDC5, that is released into the blood circulation (Bostrom et al., 2012; Erickson, 2013). In parallel to the increased PGC1α levels, our results show that early after the induction of the anorexic phenotype FNDC5 protein levels are increased. Interestingly, this effect is specific for ABA animals, indeed FNDC5 protein levels are unchanged following the single FR or EXE condition, further pointing to the ABA phenotype as the trigger for the PGC1α/FNDC5 induction.

In humans, irisin levels are enhanced in individuals engaged in exercise-induced activities, such as 10 weeks of endurance exercise training (Bostrom et al., 2012; Huh et al., 2012), and progressively reduced in those less active and sedentary (Arhire et al., 2019). Moreover, in

humans, the reduction in irisin concentrations with increased energy intake is consistent with the detrimental metabolic effects of overeating (Schlogl et al., 2015).

Plasma levels of irisin measured in our conditions, thus after 4 days of physical activity and food restriction, are slightly induced in ABA animals and slightly reduced after recovery. It has been demonstrated that in animals, plasma irisin levels increase by 65% after 3 weeks of freewheel running, while in healthy humans irisin levels double after 10 weeks of endurance exercise (Bostrom et al., 2012). Thus, our results prompt us to believe that our model might affect the PGC1α/FNDC5-Irisin axis, but still, we are aware of the limitations regarding the characterization of FNDC5-Irisin antibody, as widely described by Erickson (Erickson, 2013), and thus of the technical problems concerning the ELISA kit used to detect irisin in plasma that may lack specificity. In this regard, we are now considering increasing the sample size of the analysis, and to test irisin levels in a time-course experiment (thus evaluating earlier time points during the induction of the anorexic phenotype) to better understand Irisin modulation in our experimental conditions.

According to the literature, it has been demonstrated that hippocampal BDNF can be modulated in response of the PGC1α/FNDC5-Irisin pathway activation following exercise (Lourenco et al., 2019). Our data indicate that *Bdnf* gene expression in the Hip is induced following exercise condition at PND42 in both EXE and ABA group, suggesting that *Bdnf* induction is independent from the food restriction condition. Of note, BDNF protein translation is induced following exercise measured at PND42, an effect already reported in the literature (Wrann et al., 2013), while it is interesting that in ABA rats mBDNF expression is reduced in respect to the EXE group, suggesting that food restriction does not affect BDNF expression per se, but low energy intake is able to alter mBDNF protein levels only when combined with physical activity. Despite the exact mechanisms behind the modulation of BDNF by the PGC1α/FNDC5-Irisin pathway is mostly unknown, the observed effect on mBDNF in our model, could play a (mal)adaptive role in reward-related learning processes, thus associating a positive and pleasant effects to a condition of caloric restriction and exaggerated running, similarly to what characterize addictive behavior (Barker et al., 2015; Pitts et al., 2016) that might be a key factor in the reiteration of the typical AN aberrant behaviors.

Moreover, it appears from our results, that after 7-days of refeeding *Bdnf* gene expression, as well as BDNF protein, levels are significantly reduced in ABA rodents, highlighting potential long-lasting alterations that persist even after a period of recovery from the induction of the disease. Interestingly, this effect might be representative of a pathological state since downregulation of BDNF in the mPFC and Hip has been found in animal models of depression

and in depressed patients (Autry & Monteggia, 2012). Therefore, these findings may suggest that the combination of dietary restriction and exaggerated physical activity is able to induce a depressive-like phenotype in recovered animals, probably due to the lack of trophic supply, physiologically provided by BDNF, at the neuronal level and a decreased synaptic plasticity.

The high-affinity receptor for BDNF TrkB is indispensable for the survival, development and synaptic plasticity of several subtypes of neurons in the nervous system (Gupta et al., 2013). The anorexic phenotype induced a reduction in the phosphorylation levels of the TrkB receptor in tyrosine 706 specifically in ABA rats, a readout of receptor activation and of their downstream pathways' induction. The combination of food restriction and intense physical activity impacts TrkB receptor activation, potentially affecting also the BDNF-mediated downstream signaling pathway. Of note, the reduced levels of pTrkB in Tyr706 observed in the crude membrane fraction of hippocampal tissues persist even after the recovery period. In addition, these results are paralleled by reduced levels of the TrkB receptor total protein both at the acute phase and after the seven days period of recovery, further pointing to long-lasting alterations of the BDNF-dependent signal. Considering the relevance that BDNF has in the regulation of synaptic structural plasticity and functionality, these findings turned us to hypothesize that ABA-induced alterations in the BDNF system might have an impact also on the structural condition of the synapse. Thus, to investigate in depth ABA-induced potential alterations in structural plasticity, we measured PSD95 protein levels in Hip membrane fraction. PSD95 is the most abundant postsynaptic density protein in the brain, that has been shown to play crucial roles in maintaining synaptic structure and strength, specifically of excitatory synapses (Chen et al., 2015), and it is widely used as an indicator of synaptic integrity. Interestingly, we observed that only the combination of caloric restriction and exercise induced a marked reduction of PSD95 protein levels, a long-lasting effect that is still present even after recovery. Of note, spine density analysis performed in the Hip of ABA rodents at both time points revealed a reduced spine density in the acute phase of the disease, an effect that was paralleled by reduced numbers of mature mushroom-shaped active spines, in favor of an increase of the immature thin-shaped spines, at both time points. After recovery, even though we didn't observe any effect in terms of dendritic spine density, the reduced percentage of mature mushroom-shaped spines was still present. This effect is paralleled by increased mushroom-type head width, an effect that might be considered as a compensatory mechanism carried out to try to compensate for the reduced percentage of active spines, which serve for a proper synaptic transmission.

Evidence exists that impairments in the BDNF system might impact cognitive processes (Mottarlini et al., 2020; Zagrebelsky et al., 2020), in particular in the Hip (Vaynman et al., 2004). Notably, we observed that ABA rats, early after the induction of the anorexic phenotypes show a significant impairment in the spatial memory, typically controlled by the Hip, failing their performance in the Spatial order object recognition (SOOR) test. This result suggest that ABA rodents are not able to distinguish for objects displaced in the space, further indicating the strong impact of the combination of food restriction and intense physical exercise on cognitive processes. Overall, this ABA-induced dysregulation is long-lasting and persist even in recovered ABA rats, underlying AN-related vulnerability and potentially opening a window for relapse.

Taken together these results support the hypothesis of a crosstalk between peripheral signal and central responses, even though a causative relationship and a mechanistic description remain to be established. Despite the understanding of the physiological role of Irisin is overall scars, most of its implications in different pathological condition (physical exercise-related disease or metabolic disorders) need to be elucidated, and many studies provide still inconsistent and controversial results, this myokine, as a chemical messenger of peripheral signals to the brain, remain of interest and a valuable promising target for pharmacological intervention.

5 Conclusions

This PhD project was focused on unravelling the biological mechanisms that may play a crucial role in the maintenance of AN-induced aberrant behaviors, and in the molecular vulnerability traits that might persist even when body weight is restored, spanning from peripheral alterations to central dysfunctions.

Clinical studies report that individuals with AN show brain volumetric abnormalities and maladaptive reorganization of neural circuits within reward-sensitive brain regions (Frank et al., 2013; Titova et al., 2013) in combination with dysregulations in peripheral organ systems, circulating hormones and neuropeptides (Tortorella et al., 2014), linking peripheral dysfunctions to brain alterations. Moreover, considering the strong impact that physical exercise has in regulating energy balance and overall, in the pathophysiology of AN, skeletal muscles, and circulating myokines come into play, breeding the hypothesis of a neurometabolic axis as critical factor in the onset and maintenance of anorexic behaviors. Thus, the main goal was to expand the knowledge about the molecular mechanisms that might be involved in the pathogenesis of AN, via an in-depth characterization of a periphery-to-brain axis that might contribute to AN during a critical developmental period of life, the adolescence, taking advantage of the gold-standard animal model in the field, the activity-based anorexia (ABA) rat model. This model mimics the core symptoms of the human disease in rodents, in particular restricted caloric intake, hyperactivity, and reduced body weight, and even though we are aware that no animal model can mimic all aspects of a complex psychiatric disorder, such as AN, the ABA model has the potential to provide useful insights into the mechanisms underlying the maintenance of these maladaptive behaviors. Specifically, it allows the exploration of AN-induced neuroplastic molecular mechanisms that might help in shedding light on the neurobiological basis of AN.

Our findings indicate that adolescent female rodents exposed to a restricted diet and physical exercise reduced their food intake and their body weight, developing hyperactive and compulsive wheel running behaviors. We found that adolescent ABA rats reduced food intake and progressively increased their physical activity leading to self-starvation and hyperactivity, further aggravating weight loss, as previously observed (Chowdhury et al., 2015; Routtenberg & Kuznesof, 1967). The impact of free access to a running wheel on body weight became evident during the food restriction period only: rats exposed to a schedule of food restriction, exhibited limited weight loss compared to rats exposed to the combination of both conditions,

despite similar food intake, pointing to hyperactivity as a key driving force to starvation (Adan et al., 2011). Unlike exercise animals that maintained a voluntary and stable activity during the entire experiment, when food restriction began, wheel activity of ABA rats constantly increased over days, as expected in this model (Chen et al., 2020; Scherma et al., 2017). At the end of the recovery period, the body weight of both FR and ABA rats were restored back to control levels; however, ABA rats, despite a higher food intake compared to FR rats, took more time to restore their body weight, underlying the strenuous and persistent impact of the intense physical activity in a condition of low food intake. Of note, the maladaptive cycle of self-starvation and hyperactivity in ABA rats escalates rapidly and severely during adolescence, in agreement with the age-dependent risk for clinical AN described in the DSM-5 (DSM-V, 2013).

From a neurobiological point of view, we have shown that the induction of the ABA phenotype, but not food restriction or exercise conditions alone, causes a long-lasting increase of the GluA1/A2 ratio in the NAc crude membrane fraction, an index of GluA2-lacking Ca^{2+}-permeable AMPA (CP-AMPA) receptors formation (Reimers et al., 2011), that persist despite body-weight recovery, and an increase of the membrane levels of the NMDA GluN2A/2B subunit ratio, instead reduced after body weight recovery. These findings support the presence of an overall receptor subunit reorganization of the glutamatergic synapse at the achievement of the anorexic phenotype that correlates with ABA-induced hyperactive behavior, further corroborating the AN-induced weakening of synaptic strength in the NAc. Moreover, our data demonstrated that the ABA condition alters the homeostasis of the glutamatergic system also in the mPFC, a site of integration of numerous stimuli with a key role in converting them into efferent signals that contribute toward executive functioning, cognitive flexibility, memory, and reward-related response. In fact, ABA female rats exhibit altered molecular composition of the glutamate synapse, in terms of AMPA and NMDA receptors, and reduced dendritic spine density in the mPFC, a combined mechanism that might contribute to explain their memory impairment observed in the TOOR test. Moreover, seven days after the achievement of the anorexic phenotype, despite the body weight recovery and the mPFC structure normalized, ABA rats still displayed altered excitatory signaling and impaired temporal memory abilities. Taken together, the complex set of structural and molecular analyses herein shown point to (mal)adaptive glutamatergic rearrangements occurring in the adolescent brain, that might contribute to alter the incentive motivational system and recency discrimination abilities to drive maladaptive behaviors in AN, as revealed by the significant correlations between glutamatergic receptors and recency discrimination index with ABA-induced hyperactive

behavior and body weight loss. These events are functionally relevant since the long-term failure in recruiting critical glutamate determinants at synaptic level might reduce the transition to active dendritic spines, leading to a functional destabilization of the mPFC and conferring greater vulnerability to AN patients.

These findings may help to explain, at least in part, how an imbalance in energy intake, due to the combination of caloric restriction and hyperactivity, affects the mesocorticolimbic reward circuit in the brain, strengthening the hypothesis of an imbalance between reward and inhibition mechanisms as the potential driving force that might sustain the perpetuation of aberrant behaviors in the vicious cycle of AN. The deviation from normal brain development, and particularly the impairment in the structure and composition of the excitatory synapse in the NAc and in the mPFC, reflective of a pathological state and not of a generalized effect of malnutrition or exercise, might contribute to sustain the maintenance of aberrant behaviors, and establish a vicious cycle, perpetuating restraint over-eating and other AN-related behaviors.

In parallel to the ABA-induced glutamate system dysregulation, our findings revealed an alteration of the muscle-to-brain axis PGC1α/FNDC5/Irisin/BDNF pathway. At the achievement of the anorexic phenotype, we observed a hyperactivation of the axis at peripheral level, as shown by increased PGC1α and FNDC5 protein levels in muscle tissues and increased Irisin plasma levels, and a dysregulation of BDNF expression in the Hip, as shown by increased BDNF transcription and reduced protein translation. Interestingly, despite body weight recovery normalized the peripheral counterpart of the axis, the hippocampal reduction of BDNF still persisted. Considering the role of BDNF in the CNS, the herein observed reduction suggest that the combination of low energy intake and hyperactivity reduced hippocampal neuroplastic activity, diminishing its trophic support in the hip, and may contribute to induce a depressive-like phenotype, typical of AN patients. Overall, these findings point towards the dysregulation of the PGC1a/FNDC5/Irisin axis as potentially responsible of BDNF modulation in the brain, further corroborating the hypothesis of a periphery-to-brain crosstalk in which peripheral signals might modulate central responses. Moreover, similarly to mPFC, the anorexic phenotype is able to alter the structure of the hip, since we observed reduced spine density and morphological alterations in hippocampal dendritic spines, suggesting that the AN condition might also compromise synaptic neurotransmission in a brain region fundamental for associative reward learning. These events are functionally relevant since the discrimination index in the spatial order object recognition test, which requires an intact and functional hip,

was impaired in ABA rats at both the achievement of the anorexic phenotype and after body weight recovery.

In summary, we can conclude that the induction of anorexic phenotype in rodents, by means of the ABA model, is able to reproduce the critical symptoms of the human disease, and that this condition induces enduring maladaptive peripheral and neuroplastic alterations that involve two of the most relevant neural systems, such as the excitatory glutamatergic transmission and the BDNF system. Altogether the herein shown AN-induced dysregulation in the brain, coupled with peripheral alterations, may sustain, at least in part, the motivational mechanisms behind the reiteration of the aberrant behaviors typical of the disease, and might be the trigger that could lead, in turn, to cognitive dysfunction, which is consistently observed in AN patients. In this scenario, our data might pave the way toward new pharmacotherapies designed to manipulate these systems and to restore normal cognitive functions. Finally, given the neurobiological differences that we observed between the acute phase of the AN phenotype and after recovery, future studies will try to get further insights into the mechanisms that govern the difference between these two phases of the disorder.

6 Tables and statistics

Results of chapter 4.1

Two-way ANOVA	Body weight (%)		Food intake (g)		Distance travelled (m)	
	F (DFn, DFd)	*P value*	*F (DFn, DFd)*	*P value*	*F (DFn, DFd)*	*P value*
Time x treatment	F (54, 360) = 10,54	P<0,0001	F (39, 260) = 32,63	P<0,0001	F (6, 174) = 6,852	P<0,0001
Time	F (18, 360) = 475,5	P<0,0001	F (5,399, 108,0) = 130,2	P<0,0001	F (6, 174) = 41,91	P<0,0001
Food restriction	F (3, 20) = 9,131	P=0,0005	F (3, 20) = 5,316	P=0,0074	F (1, 29) = 20,13	P=0,0001
Exercise	F (20, 360) = 18,35	P<0,0001	F (20, 260) = 5,568	P<0,0001	F (29, 174) = 2,615	P<0,0001

	Mean speed (m/min)		Max speed (m/min)		Long-exercise sequence	
	F (DFn, DFd)	*P value*	*F (DFn, DFd)*	*P value*	*F (DFn, DFd)*	*P value*
Time x treatment	F (6, 126) = 3,113	P=0,0071	F (6, 126) = 10,93	P<0,0001	F (6, 126) = 4,681	P=0,0002
Time	F (6, 126) = 39,90	P<0,0001	F (6, 126) = 21,27	P<0,0001	F (6, 126) = 12,05	P<0,0001
Food restriction	F (1, 21) = 4,334	P=0,0498	F (1, 21) = 4,948	P=0,0372	F (1, 21) = 4,113	P=0,0554
Exercise	F (21, 126) = 5,767	P<0,0001	F (21, 126) = 10,90	P<0,0001	F (21, 126) = 2,932	P=0,0001

Table 9 Repeated measures two-way ANOVA analysis of body weight, food intake, distance travelled, mean speed, maximum speed and long-exercise sequences presented in Figures 14 and 15.

Body weight	Bonferroni's multiple comparisons test	
	Mean difference (g)	Adjusted p value
pnd39		
Control vs. Food restriction	18,92	0,0066
Control vs. Activity-based anorexia	22,67	0,0002
Exercise vs. Activity-based anorexia	16,60	0,0461
pnd40		
Control vs. Food restriction	30,25	<0,0001
Control vs. Activity-based anorexia	35,29	<0,0001
Food restriction vs. Exercise	-23,37	<0,0001
Exercise vs. Activity-based anorexia	28,42	<0,0001
pnd41		
Control vs. Food restriction	36,52	<0,0001
Control vs. Activity-based anorexia	44,28	<0,0001
Food restriction vs. Exercise	-30,54	<0,0001
Exercise vs. Activity-based anorexia	38,30	<0,0001
pnd42		
Control vs. Food restriction	37,03	<0,0001
Control vs. Activity-based anorexia	54,81	<0,0001
Food restriction vs. Exercise	-31,43	<0,0001
Food restriction vs. Activity-based anorexia	17,79	0,0174
Exercise vs. Activity-based anorexia	49,22	<0,0001
pnd43		
Control vs. Food restriction	17,62	0,0199
Control vs. Exercise	1,663	>0,9999
Control vs. Activity-based anorexia	22,90	0,0001
Food restriction vs. Activity-based anorexia	5,280	>0,9999
Exercise vs. Activity-based anorexia	21,24	0,0008
pnd44		
Control vs. Exercise	2,224	>0,9999
Control vs. Activity-based anorexia	19,86	0,0028
Food restriction vs. Exercise	-11,96	>0,9999
Food restriction vs. Activity-based anorexia	5,681	>0,9999
Exercise vs. Activity-based anorexia	17,64	0,0197
pnd45		
Control vs. Exercise	0,7040	>0,9999
Control vs. Activity-based anorexia	16,76	0,0405

Food intake	Bonferroni's multiple comparisons test	
	Mean difference (g)	Adjusted p value
pnd36		
Control vs. Exercise	3,583	0,0358
pnd39		
Control vs. Food restriction	15,06	<0,0001
Control vs. Activity-based anorexia	14,88	<0,0001
Food restriction vs. Exercise	-15,14	<0,0001
Exercise vs. Activity-based anorexia	14,96	<0,0001
pnd40		
Control vs. Food restriction	13,36	<0,0001
Control vs. Activity-based anorexia	12,33	<0,0001
Food restriction vs. Exercise	-13,15	<0,0001
Exercise vs. Activity-based anorexia	12,13	<0,0001
pnd41		
Control vs. Food restriction	11,52	<0,0001
Control vs. Activity-based anorexia	10,40	<0,0001
Food restriction vs. Exercise	-13,83	<0,0001
Exercise vs. Activity-based anorexia	12,71	<0,0001
pnd42		
Control vs. Food restriction	10,77	<0,0001
Control vs. Activity-based anorexia	10,19	0,0003
Food restriction vs. Exercise	-12,63	<0,0001
Exercise vs. Activity-based anorexia	12,04	<0,0001
pnd43		
Control vs. Food restriction	-6,167	0,0095
pnd45		
Control vs. Food restriction	-4,667	0,0083
pnd46		
Control vs. Activity-based anorexia	-6,083	0,0222
pnd47		
Control vs. Activity-based anorexia	-7,917	0,0073
Exercise vs. Activity-based anorexia	-6,333	0,0038
pnd48		
Control vs. Activity-based anorexia	-4,250	0,0271

Table 10 Detailed statistical values of data presented in Figure 14. Mean differences and adjusted *p* values relative to Bonferroni's multiple comparisons test are presented for the analysis of body weight (Fig 14 a) and food intake (Fig 14 b)

Distance travelled	Bonferroni's multiple comparisons test	
	Mean difference (m)	Adjusted p value
Exercise - Activity-based anorexia		
pnd40	-3877	<0,0001
pnd41	-4510	<0,0001
pnd42	-4351	<0,0001

Long-exercise sequences	Bonferroni's multiple comparisons test
	Adjusted p value
Exercise - Activity-based anorexia	
pnd41	0,0241
pnd42	0,0001

Mean velocity	Bonferroni's multiple comparisons test
	Adjusted p value
Exercise - Activity-based anorexia	
pnd41	0,0031
pnd42	0,0201

Max velocity	Bonferroni's multiple comparisons test
	Adjusted p value
Exercise - Activity-based anorexia	
pnd41	0,0333
pnd42	<0,0001

Table 11 Detailed statistical values of data presented in Figure 15. Mean differences and adjusted *p* values relative to Bonferroni's multiple comparisons test are presented for the analysis of distance travelled (Fig 15 a), mean speed (Fig 15 b), maximum speed (Fig 15 c) and long-exercise sequence (Fig 15 e)

	Two-way ANOVA	Whole homogenate – Acute phase	
		F (DFn, DFd)	P value
NR2A	Interaction	F (1, 19) = 1,431	P=0,2464
	Exercise	F (1, 19) = 12,25	P=0,0024
	Food-restriction	F (1, 19) = 0,2040	P=0,6567
NR2B	Interaction	F (1, 19) = 1,377	P=0,2552
	Exercise	F (1, 19) = 8,237	P=0,0098
	Food-restriction	F (1, 19) = 1,014	P=0,3266
GRIP	Interaction	F (1, 19) = 5,600	P=0,0287
	Exercise	F (1, 19) = 5,535	P=0,0296
	Food-restriction	F (1, 19) = 1,602	P=0,2209
GluA1	Interaction	F (1, 19) = 0,6317	P=0,4365
	Exercise	F (1, 19) = 0,9214	P=0,3492
	Food-restriction	F (1, 19) = 0,006563	P=0,9363
GluA2	Interaction	F (1, 19) = 2,761	P=0,1130
	Exercise	F (1, 19) = 5,560	P=0,0292
	Food-restriction	F (1, 19) = 0,002572	P=0,9601
SAP102	Interaction	F (1, 19) = 1,004	P=0,3289
	Exercise	F (1, 19) = 7,228	P=0,0145
	Food-restriction	F (1, 19) = 0,0005493	P=0,9815
SAP97	Interaction	F (1, 19) = 4,999	P=0,0376
	Exercise	F (1, 19) = 7,629	P=0,0124
	Food-restriction	F (1, 19) = 1,096	P=0,3083
PSD95	Interaction	F (1, 19) = 0,3133	P=0,5822
	Exercise	F (1, 19) = 0,08531	P=0,7734
	Food-restriction	F (1, 19) = 4,535	P=0,0465

	Two-way ANOVA	Whole homogenate – Recovery phase	
		F (DFn, DFd)	P value
NR2A	Interaction	F (1, 20) = 22,44	P=0,0001
	Exercise	F (1, 20) = 11,66	P=0,0027
	Food-restriction	F (1, 20) = 9,209	P=0,0065
NR2B	Interaction	F (1, 20) = 28,01	P<0,0001
	Exercise	F (1, 20) = 30,03	P<0,0001
	Food-restriction	F (1, 20) = 27,06	P<0,0001
GRIP	Interaction	F (1, 20) = 0,9104	P=0,3514
	Exercise	F (1, 20) = 3,692	P=0,0690
	Food-restriction	F (1, 20) = 0,5258	P=0,4768
GluA1	Interaction	F (1, 20) = 20,19	P=0,0002
	Exercise	F (1, 20) = 0,3298	P=0,5722
	Food-restriction	F (1, 20) = 3,079	P=0,0946
GluA2	Interaction	F (1, 20) = 1,697	P=0,2074
	Exercise	F (1, 20) = 0,6153	P=0,4420
	Food-restriction	F (1, 20) = 7,024	P=0,0154
SAP102	Interaction	F (1, 20) = 0,003614	P=0,9527
	Exercise	F (1, 20) = 6,177	P=0,0219
	Food-restriction	F (1, 20) = 0,07693	P=0,7843
SAP97	Interaction	F (1, 20) = 0,008498	P=0,9275
	Exercise	F (1, 20) = 4,665	P=0,0431
	Food-restriction	F (1, 20) = 1,885	P=0,1849
PSD95	Interaction	F (1, 20) = 12,40	P=0,0021
	Exercise	F (1, 20) = 3,846	P=0,0640
	Food-restriction	F (1, 20) = 6,445	P=0,0195

Table 12 Two-way ANOVA analysis of protein expression data measured in the whole homogenate of the NAc in the acute phase of the pathology (top) and after a 7-days recovery period (bottom) presented in Figures 16–2.

	Two-way ANOVA	Crude membrane fraction – Acute phase	
		F (DFn, DFd)	P value
NR2A	Interaction	F (1, 17) = 17,12	P=0,0007
	Exercise	F (1, 17) = 26,07	P<0,0001
	Food-restriction	F (1, 17) = 32,25	P<0,0001
NR2B	Interaction	F (1, 18) = 15,78	P=0,0009
	Exercise	F (1, 18) = 2,986	P=0,1011
	Food-restriction	F (1, 18) = 0,5549	P=0,4659
GRIP	Interaction	F (1, 19) = 3,625	P=0,0722
	Exercise	F (1, 19) = 0,1049	P=0,7495
	Food-restriction	F (1, 19) = 2,240	P=0,1509
GluA1	Interaction	F (1, 18) = 2,970	P=0,1020
	Exercise	F (1, 18) = 3,913	P=0,0634
	Food-restriction	F (1, 18) = 6,350	P=0,0214
GluA2	Interaction	F (1, 19) = 35,47	P<0,0001
	Exercise	F (1, 19) = 0,9781	P=0,3351
	Food-restriction	F (1, 19) = 0,6139	P=0,4430
SAP102	Interaction	F (1, 18) = 5,283	P=0,0337
	Exercise	F (1, 18) = 0,003415	P=0,9540
	Food-restriction	F (1, 18) = 5,000	P=0,0382
SAP97	Interaction	F (1, 18) = 10,99	P=0,0039
	Exercise	F (1, 18) = 4,814	P=0,0416
	Food-restriction	F (1, 18) = 0,7498	P=0,3979
PSD95	Interaction	F (1, 18) = 2,580	P=0,1257
	Exercise	F (1, 18) = 1,272	P=0,2741
	Food-restriction	F (1, 18) = 0,0009369	P=0,9759

	Two-way ANOVA	Crude membrane fraction – Recovery phase	
		F (DFn, DFd)	P value
NR2A	Interaction	F (1, 20) = 22,44	P=0,0001
	Exercise	F (1, 20) = 11,66	P=0,0027
	Food-restriction	F (1, 20) = 9,209	P=0,0065
NR2B	Interaction	F (1, 20) = 28,01	P<0,0001
	Exercise	F (1, 20) = 30,03	P<0,0001
	Food-restriction	F (1, 20) = 27,06	P<0,0001
GRIP	Interaction	F (1, 19) = 6,378	P=0,0206
	Exercise	F (1, 19) = 26,20	P<0,0001
	Food-restriction	F (1, 19) = 3,669	P=0,0706
GluA1	Interaction	F (1, 20) = 20,19	P=0,0002
	Exercise	F (1, 20) = 0,3298	P=0,5722
	Food-restriction	F (1, 20) = 3,079	P=0,0946
GluA2	Interaction	F (1, 20) = 1,697	P=0,2074
	Exercise	F (1, 20) = 0,6153	P=0,4420
	Food-restriction	F (1, 20) = 7,024	P=0,0154
SAP102	Interaction	F (1, 20) = 4,475	P=0,0471
	Exercise	F (1, 20) = 4,731	P=0,0418
	Food-restriction	F (1, 20) = 1,651	P=0,2135
SAP97	Interaction	F (1, 20) = 8,141	P=0,0098
	Exercise	F (1, 20) = 20,05	P=0,0002
	Food-restriction	F (1, 20) = 1,890	P=0,1844
PSD95	Interaction	F (1, 19) = 11,28	P=0,0033
	Exercise	F (1, 19) = 5,877	P=0,0255
	Food-restriction	F (1, 19) = 3,221	P=0,0886

Table 13 Two-way ANOVA analysis of protein expression data measured in the crude membrane fraction of the NAc in the acute phase of the pathology (left) and after a 7-days recovery period (right) presented in Figures 16–21.

Results of chapter 4.2

Body weight	Bonferroni's multiple comparisons test	
	Mean difference (g)	Adjusted p value
pnd39		
CTRL vs. FR	21,32	< 0,0001
CTRL vs. ABA	21,75	< 0,0001
pnd40		
CTRL vs. FR	33,43	< 0,0001
CTRL vs. ABA	39,63	< 0,0001
FR vs. EXE	-22,60	0,0002
EXE vs. ABA	28,79	< 0,0001
pnd41		
CTRL vs. FR	41,39	< 0,0001
CTRL vs. ABA	49,18	< 0,0001
FR vs. EXE	-31,17	< 0,0001
EXE vs. ABA	38,95	< 0,0001
pnd42		
CTRL vs. FR	42,85	< 0,0001
CTRL vs. ABA	58,61	< 0,0001
FR vs. EXE	-32,86	< 0,0001
FR vs. ABA	15,76	0,0221
EXE vs. ABA	48,62	< 0,0001
pnd43		
CTRL vs. FR	24,84	< 0,0001
CTRL vs. ABA	30,22	< 0,0001
EXE vs. ABA	21,34	< 0,0001
pnd44		
CTRL vs. FR	21,77	< 0,0001
CTRL vs. ABA	27,60	< 0,0001
EXE vs. ABA	17,79	0,0031
pnd45		
CTRL vs. FR	20,12	0,0002
CTRL vs. ABA	24,11	< 0,0001
EXE vs. ABA	15,79	0,0216
pnd46		
CTRL vs. FR	18,36	0,0017
CTRL vs. ABA	20,67	< 0,0001
pnd47		
CTRL vs. FR	15,30	0,0339
CTRL vs. ABA	15,59	0,0025
pnd48		
CTRL vs. ABA	13,78	0,0193

Food intake	Bonferroni's multiple comparisons test	
	Mean difference (g)	Adjusted p value
pnd39		
CTRL vs. FR	13,98	<0,0001
CTRL vs. ABA	15,17	<0,0001
FR vs. EXE	-15,14	<0,0001
EXE vs. ABA	16,33	<0,0001
pnd40		
CTRL vs. FR	11,87	<0,0001
CTRL vs. ABA	11,76	<0,0001
FR vs. EXE	-13,15	<0,0001
EXE vs. ABA	13,04	<0,0001
pnd41		
CTRL vs. FR	11,54	<0,0001
CTRL vs. ABA	9,759	<0,0001
FR vs. EXE	-13,83	<0,0001
EXE vs. ABA	12,05	<0,0001
pnd42		
CTRL vs. FR	9,827	<0,0001
CTRL vs. ABA	10,71	<0,0001
FR vs. EXE	-12,63	<0,0001
EXE vs. ABA	13,51	<0,0001
pnd43		
CTRL vs. FR	-5,570	<0,0001
CTRL vs. EXE	-3,903	0,0079
CTRL vs. ABA	-4,645	<0,0001
pnd45		
CTRL vs. FR	-4,677	0,0003
CTRL vs. ABA	-3,060	0,0335
pnd46		
CTRL vs. FR	-5,260	<0,0001
CTRL vs. ABA	-4,410	<0,0001
pnd47		
CTRL vs. FR	-4,287	0,0016
CTRL vs. ABA	-5,320	<0,0001
EXE vs. ABA	-3,950	0,0065
pnd48		
CTRL vs. ABA	-3,160	0,0216

Distance travelled	Bonferroni's multiple comparisons test	
EXE vs. ABA	Mean difference (m)	Adjusted p value
pnd40	-3877	<0,0001
pnd41	-4510	<0,0001
pnd42	-4351	<0,0001

Table 14 Detailed statistical values of data presented in Figure 23. Mean differences and adjusted *p* values relative to Bonferroni's multiple comparisons test are presented for the analysis of body weight (Fig 23 a), food intake (Fig 23 b) and distance travelled (Fig 23 c).

		Three-way ANOVA (followed by Tuckey's post hoc test)	
Whole homogenate		**F value**	**p value**
GluA1	Time	F (1, 38) = 6,646	P=0,0139
	Food restriction	F (1, 38) = 15,25	P=0,0004
	Time x Food restriction	F (1, 38) = 5,905	P=0,0199
	Exercise x Food restriction	F (1, 38) = 16,34	P=0,0002
	Time x Exercise x Food restriction	F (1, 38) = 10,71	P=0,0023
GluA2	Food restriction	F (1, 38) = 6,167	P=0,0175
	Time x Food restriction	F (1, 38) = 6,442	P=0,0154
	Time x Exercise x Food restriction	F (1, 38) = 7,755	P=0,0083
GluN2B	Time	F (1, 38) = 67,83	P<0,0001
	Food restriction	F (1, 38) = 17,32	P=0,0002
	Time x Food restriction	F (1, 38) = 5,586	P=0,0233
	Exercise x Food restriction	F (1, 38) = 18,71	P=0,0001
	Time x Exercise x Food restriction	F (1, 38) = 9,666	P=0,0035
GluN1	Time	F (1, 39) = 27,76	P<0,0001
	Time x Food restriction	F (1, 39) = 20,36	P<0,0001
	Exercise x Food restriction	F (1, 39) = 13,17	P=0,0008
	Time x Exercise x Food restriction	F (1, 39) = 7,280	P=0,0102
SAP97	Time	F (1, 38) = 22,04	P<0,0001
	Food restriction	F (1, 38) = 8,992	P=0,0048
	Time x Exercise	F (1, 38) = 4,191	P=0,0476
	Time x Food restriction	F (1, 38) = 11,21	P=0,0018
	Time x Exercise x Food restriction	F (1, 38) = 6,051	P=0,0186
F-actin	Time	F (1, 39) = 40,91	P<0,0001
	Time x Exercise	F (1, 39) = 12,32	P=0,0011
	Time x Food restriction	F (1, 39) = 4,550	P=0,0393
	Exercise x Food restriction	F (1, 39) = 20,27	P<0,0001
	Time x Exercise x Food restriction	F (1, 39) = 5,729	P=0,0216
Neuroligin	Time	F (1, 39) = 5,025	P=0,0307
	Time x Food restriction	F (1, 39) = 6,209	P=0,0171
	Time x Exercise x Food restriction	F (1, 39) = 8,002	P=0,0073

Table 15 Effects of FR, EXE and ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery period (PND49) on the expression of critical determinants of the glutamate synapse, measured in the whole homogenate of the mPFC, and presented in Table 7. Three-way ANOVA main effects with F and adjusted p values are presented.

		Three-way ANOVA (followed by Tuckey's post hoc test)	
Post-synaptic density		**F value**	**p value**
GluA2	Time	F (1, 39) = 9,347	P=0,0040
	Food restriction	F (1, 39) = 4,205	P=0,0471
	Time x Exercise	F (1, 39) = 5,046	P=0,0304
	Time x Food restriction	F (1, 39) = 29,72	P<0,0001
	Time x Exercise x Food restriction	F (1, 39) = 40,49	P<0,0001
GluN2A	Exercise	F (1, 39) = 13,32	P=0,0008
	Time x Exercise	F (1, 39) = 11,78	P=0,0014
	Exercise x Food restriction	F (1, 39) = 5,423	P=0,0252
	Time x Exercise x Food restriction	F (1, 39) = 19,17	P<0,0001
GluN2B	Time	F (1, 38) = 6,789	P=0,0130
	Exercise	F (1, 38) = 5,350	P=0,0262
	Time x Food restriction	F (1, 38) = 6,268	P=0,0167
	Exercise x Food restriction	F (1, 38) = 6,630	P=0,0140
	Time x Exercise x Food restriction	F (1, 38) = 29,69	P<0,0001
SAP102	Exercise	F (1, 38) = 14,06	P=0,0006
	Time x Food restriction	F (1, 38) = 4,387	P=0,0429
	Exercise x Food restriction	F (1, 38) = 10,48	P=0,0025
	Time x Exercise x Food restriction	F (1, 38) = 34,22	P<0,0001
SAP97	Time x Exercise	F (1, 39) = 13,60	P=0,0007
	Time x Exercise x Food restriction	F (1, 39) = 16,22	P=0,0003
F-actin	Time x Exercise	F (1, 39) = 21,56	P<0,0001
	Time x Food restriction	F (1, 39) = 13,67	P=0,0007
	Time x Exercise x Food restriction	F (1, 39) = 10,94	P=0,0020
Neuroligin	Time	F (1, 38) = 27,74	P<0,0001
	Exercise	F (1, 38) = 42,55	P<0,0001
	Time x Exercise	F (1, 38) = 8,454	P=0,0060
	Time x Exercise x Food restriction	F (1, 38) = 6,339	P=0,0161
PSD95	Time	F (1, 38) = 19,86	P<0,0001
	Time x Exercise x Food restriction	F (1, 38) = 5,704	P=0,0220

Table 16 Effects of FR, EXE and ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery period (PND49) on the expression of critical determinants of the glutamate synapse, measured in the post synaptic density fraction of the mPFC, and presented in Figures 24-26. Three-way ANOVA main effects with F and adjusted p values are presented.

			Two-way ANOVA (followed by Tuckey's post hoc test)	
	Whole homogenate		**F value**	**p value**
Acute Phase	**GluN2A**	Exercise x Food restriction	F (1, 19) = 12,86	P=0,0020
		Exercise	F (1, 19) = 13,81	P=0,0015
	SAP102	Food restriction	F (1, 19) = 13,89	P=0,0017
	PSD95	Food restriction	F (1, 19) = 14,44	P=0,0012
	N-cadherin	Exercise x Food restriction	F (1, 19) = 6,65	P=0,0184
After recovery	**GluN2A**	Exercise x Food restriction	F (1, 20) = 4,549	P=0,0455
	N-cadherin	Exercise	F (1, 20) = 5,932	P=0,0243
	Post-synaptic density		**F value**	**p value**
Acute Phase	**GluA1**	Exercise x Food restriction	F (1, 19) = 4,850	P=0,0402
After recovery	**GluA1**	Exercise x Food restriction	F (1, 20) = 22,60	P=0,0001
		Exercise	F (1, 20) = 51,90	P<0,0001
		Food restriction	F (1, 20) = 14,75	P=0,0010
Acute Phase	**GluN1**	Exercise x Food restriction	F (1, 18) = 11,45	P=0,0033
		Exercise	F (1, 18) = 10,38	P=0,0047
		Food restriction	F (1, 18) = 8,906	P=0,0080
After recovery	**GluN1**	Exercise x Food restriction	F (1, 19) = 8,124	P=0,0102
		Food restriction	F (1, 19) = 17,04	P=0,0006
After recovery	**N-cadherin**	Exercise x Food restriction	F (1, 19) = 8,028	P=0,0106
		Exercise	F (1, 19) = 11,90	P=0,0027
		Food restriction	F (1, 19) = 10,44	P=0,0044

Table 17 Effects of FR, EXE and ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery period (PND49) on the expression of critical determinants of the glutamate synapse, measured in the whole homogenate and in the post synaptic density fraction of the mPFC, and presented in Table 7 and Fig 24-26, respectively. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase and After recovery		Two-way ANOVA (followed by Tuckey's post hoc test)	
		F value	**p value**
Spine density	Time x ABA	F (1, 28) = 4,812	P=0,0367
	Time	F (1, 28) = 11,17	P=0,0024
	ABA	F (1, 28) = 7,023	P=0,0131
Length	Interaction	F (1, 28) = 4,468	P=0,0436
	Time	F (1, 28) = 6,510	P=0,0165
Width	Interaction	F (1, 28) = 5,096	P=0,0320
	ABA	F (1, 28) = 4,749	P=0,0379
Mushrooms	Interaction	F (1, 28) = 16,57	P=0,0003
	Time	F (1, 28) = 7,161	P=0,0123
Stubby	Time	F (1, 28) = 22,94	P<0,0001
Filopodia	Interaction	F (1, 28) = 7,161	P=0,0083

Table 18 Effects of ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery (PND49) on density and morphology of dendritic spines of adolescent female rodents as represented in Fig 27-28. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase and After recovery		Two-way ANOVA (followed by Tuckey's post hoc test)	
		F value	**p value**
Discrimination index	Time x ABA	F (1, 28) = 4,569	P=0,0414
	ABA	F (1, 28) = 28,70	P<0,0001

Table 19 Effects of ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery (PND49) on the discrimination index of the TOOR test performed in adolescent female rodents as presented in Fig 29. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase		Two-way ANOVA (followed by Tuckey's post hoc test)	
Gene		***F value***	***p value***
Glut4	Food restriction	F (1, 20) = 119,5	P<0,0001
Cpt1β	Time x Exercise x Food restriction	F (1, 20) = 61,64	P<0,0001
	Food restriction	F (1, 20) = 58,85	P<0,0001
After recovery			
Glut4	Food restriction	F (1, 20) = 5,525	P=0,0291

Table 20 Effects of FR, EXE and ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery period (PND49) on the gene expression of *Glut4* and *Cpt1β* measured in the soleus muscle of adolescent female rodents as shown in Fig 30. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase		Two-way ANOVA (followed by Tuckey's post hoc test)	
Whole homogenate		***F value***	***p value***
PGC1a	Time x Exercise x Food restriction	F (1, 20) = 4,430	P=0,0482
	Exercise	F (1, 20) = 31,14	P<0,0001
FNDC5	Time x Exercise x Food restriction	F (1, 20) = 14,48	P=0,0011
After recovery			
PGC1a	Food restriction	F (1, 20) = 10,79	P=0,0037
FNDC5	Exercise	F (1, 20) = 14,23	P=0,0012

Table 21 Effects of FR, EXE and ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery period (PND49) on the protein expression of PGC1α and FNDC5 measured in the soleus muscle whole homogenate of adolescent female rodents as shown in Fig 31. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase		Two-way ANOVA (followed by Tuckey's post hoc test)	
Gene		***F value***	***p value***
Total Bdnf	Exercise	F (1, 18) = 33,26	P<0,0001
After recovery			
Total Bdnf	Food restriction	F (1, 17) = 15,32	P=0,0011
	Exercise	F (1, 17) = 30,47	P<0,0001
	Time x Exercise x Food restriction	F (1, 17) = 9,108	P=0,0078

Table 22 Effects of FR, EXE and ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery period (PND49) on the gene expression of total *Bdnf* measured in the Hip of adolescent female rodents as shown in Fig 32. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase		Two-way ANOVA (followed by Tuckey's post hoc test)	
Crude membrane fraction		***F value***	***p value***
BDNF	Food restriction	F (1, 20) = 6,939	P=0,0159
	Exercise	F (1, 20) = 27,01	P<0,0001
	Time x Exercise x Food restriction	F (1, 20) = 9,323	P=0,0063
pTrkB y706	Food restriction	F (1, 20) = 20,54	P=0,0002
	Time x Exercise x Food restriction	F (1, 20) = 15,38	P=0,0008
TrkB	Food restriction	F (1, 20) = 15,93	P=0,0007
PSD95	Food restriction	F (1, 20) = 6,325	P=0,0206
	Exercise	F (1, 20) = 11,35	P=0,0031
	Time x Exercise x Food restriction	F (1, 20) = 4,538	P=0,0458
After recovery			
BDNF	Food restriction	F (1, 20) = 20,38	P=0,0001
pTrkB y706	Food restriction	F (1, 20) = 12,71	P=0,0019
TrkB	Food restriction	F (1, 20) = 5,128	P=0,0348
	Time x Exercise x Food restriction	F (1, 20) = 6,451	P=0,0195
PSD95	Exercise	F (1, 20) = 7,612	P=0,0121
	Time x Exercise x Food restriction	F (1, 20) = 6,749	P=0,0172

Table 23 Effects of FR, EXE and ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery period (PND49) on the protein expression of BDNF, pTrkB y706, TrkB and PSD95 measured in the soleus muscle whole homogenate of adolescent female rodents as shown in Fig 33. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase and After recovery		Two-way ANOVA (followed by Tuckey's post hoc test)	
		F value	**p value**
Spine density	Time x ABA	F (1, 28) = 4,706	P=0,0387
	ABA	F (1, 28) = 14,77	P=0,0006
Mushrooms	ABA	F (1, 28) = 10,37	P=0,0032
Thin	ABA	F (1, 28) = 20,64	P<0,0001
Mushrooms head width	Time x ABA	F (1, 28) = 2,232	P=0,0491
	ABA	F (1, 28) = 8,430	P=0,0071

Table 24 Effects of ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery (PND49) on density and morphology of dendritic spines of adolescent female rodents as shown in Fig 36. Two-way ANOVA main effects with F and adjusted p values are presented.

Acute phase and After recovery		Two-way ANOVA (followed by Tuckey's post hoc test)	
		F value	**p value**
Discrimination index	ABA	F (1, 35) = 37,34	P<0,0001

Table 25 Effects of ABA induction in the acute phase of AN, at PND42, and after a 7-days recovery (PND49) on the discrimination index of the SOOR test performed in adolescent female rodents as shown in Fig 37. Two-way ANOVA main effects with F and adjusted p values are presented.

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