EATING DISORDERS FROM BINGE TO ANOREXIA: BASIC AND CLINICAL APPROACHES FOR A TRANSLATIONAL RESEARCH

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EATING DISORDERS FROM BINGE TO ANOREXIA: BASIC AND CLINICAL APPROACHES FOR A TRANSLATIONAL RESEARCH

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Table of Contents

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EDITORIAL
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Editorial: Eating Disorders From Binge to Anorexia: Basic and Clinical Approaches for a Translational Research

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Keywords: eating behavior, hypothalamus, reward, goal-directed behavior, autonomic nervous system, animal models, epigenetic

Editorial on the Research Topic

Eating Disorders From Binge to Anorexia: Basic and Clinical Approaches for a Translational Research

Eating disorders (ED) cover a variety of psychiatric illnesses (anorexia nervosa/AN, bulimia nervosa, and binge eating) in which individuals express abnormal eating behaviors, often resulting in either insufficient or excessive food intake. The challenge is to understand how the eating behavior turns aberrant to the point that it becomes life threatening. In mammals, food intake involves both peripheral and central effectors directly linked to energy homeostasis regulation. It integrates neuronal circuitries that regulate stress, emotions, reward, biological rhythms, learning, and individual experience with food. In humans, it includes cognitive processes and socio-cultural and genetic factors. Thus, eating depends on the functioning of a hard-wired homeostatic circuitry–that is almost entirely identical between mammals because of evolutionary selection–together with a more flexible non-homeostatic hedonic circuitry, the functions of which vary according to individuals' experiences and/or epigenetic variations. Translational research, by bridging the gap between basic research and medical practice, allows for assessing the complex crosstalk between the central nervous system and peripheral organs, to better decipher the mechanisms leading to ED.

The aim of this Research Topic was to gather pioneering articles from experts in the field to better understand the multifactorial facets of ED. It is a compilation of nine original research articles, review papers, or perspectives written by 55 authors, covering pre-clinical, translational, and clinical aspects.

The phenomenology of ED relates to altered functioning of various systems, including the autonomic nervous system (ANS). As the stress response is an integral part of the ANS, the original and methodological paper of Simões-Capela et al. designed an experiment allowing the experimenter to capture ANS tonic variations and phasic activations in response to stressors in patients suffering from various ED. These devices are easy to use and can be adapted to different clinical conditions. They will help in defining a dimensional biologically-oriented nosology and establish a multiparametric model of the ED spectrum.

Two original research articles explored inter-individual variability in the susceptibility to developing ED. De Francesco et al. showed that inbred C57BL/6 mice display a large degree of inter-individual variability in high-fat consumption. Higher consumption of a high-fat diet was associated with a higher activation of mesencephalic dopamine neurons, suggesting a differential

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response to the rewarding properties of the high-fat diet in high vs. low consumers. Brenachot et al. showed that inter-individual vulnerability to developing maladaptive eating and obesity induced by the diet also depends on homeostatic circuits involving the polysialic acid-neural cell adhesion molecule, a key regulator of brain plasticity. The cross-talk between peripheral signals and hypothalamic circuits has long been shown to control hunger as well as a wide range of behaviors such as motivation, locomotor activity, negative reinforcement, and anxiety, as highlighted in the review by Méquinion et al. They underlined the potential role of hypothalamic AgRP neurons and ghrelin signaling in both the metabolic and behavioral adaptations to undernutrition in AN, a disorder with both psychiatric and metabolic etiology.

Amongst the brain modifications induced by the chronic self-induced food restriction observed in AN patients, the meta-analysis of Zhang et al. aimed to identify the most consistent white matter abnormalities between AN patients and healthy controls using ≪ seed-based d mapping ≫, a statistical technique for meta-analyzing studies that uses neuroimaging techniques to investigate the changes in brain activity or structure. This first quantitative meta-analysis revealed, in AN, microstructural differences in the interhemispheric connections and limbic association fibers.

Modeling AN in laboratory animals is a challenge since this psychiatric disorder is characterized by voluntary food restriction. Most animal models developed to date attempt to mimic core features of human AN. The systematic review of Schalla and Stengel on the Activity-Based-Anorexia (ABA) rodent model recapitulates a wide range of cellular, molecular, and behavioral changes (brain, hormonal, and immune alterations) that may underline AN. The review also highlights potential future developments such as the use of pharmacological interventions or chronic adaptations to the ABA model. Besides this "environmental" model of AN, Maltais et al. (1) described a genetic model of AN, the *anx/anx* mouse, which displays a spontaneous mutation on the chromosome 2 between the markers D2Mit133 and Jojo5 (2). The review by Nilsson depicted how this model can be considered as a valuable natural model to explore the (neuro)biology of AN and better understand their abnormal response to negative energy balance. Indeed, these mice share several characteristics with AN patients (emaciation, starvation, or pre-mature death). They also display changes in hypothalamic neuro-peptidergic and -transmitter systems that regulate food intake and metabolism, accompanied by hypothalamic inflammation and degeneration and by alterations in glucose homeostasis and pancreatic function.

However, AN is a disorder with a complex and multifactorial etiology, making it difficult to model in living organisms. Thanks to *in vitro* genomic and epigenomic analyses, identification of multiple genetic, epigenetic, and cellular bases of AN has been possible. Maussion et al. suggest to use the power of induced pluripotent stem cells combined with CRISPR–Cas9 technology as a tool to investigate new molecular and cellular mechanisms for this disorder. The authors present cellular, molecular, and whole organism models that will help to improve our current understanding of the pathophysiology of ED and provide new therapeutic strategies to address specific endophenotypes.

Finally, the review by Södersten et al. discussed current opinions about the etiology of AN and presented a successful approach to treat this disorder and achieve a low level of relapse. The review suggests that the standard perspective to consider AN, i.e., as a neurochemically and genetically mediated mental illness, needs to be adjusted because the clinical treatment classically used (cognitive therapy) is at a standstill. The authors discussed the validity of the evolutionary perspective that includes, for example, physical activity as an adaptation for food seeking. Based on a mathematical model, they explained their successful experience in treating AN using a method in which patients restore their eating behavior by practicing eating. The control of eating behavior is therefore outsourced to a machine that provides feedback on how to eat.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Lack of Hypothalamus Polysialylation Inducibility Correlates With Maladaptive Eating Behaviors and Predisposition to Obesity

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High variability exists in individual susceptibility to develop overweight in an obesogenic environment and the biological underpinnings of this heterogeneity are poorly understood. In this brief report, we show in mice that the vulnerability to diet-induced obesity is associated with low level of polysialic acid-neural cell adhesion molecule (PSA-NCAM), a factor of neural plasticity, in the hypothalamus. As we previously shown that reduction of hypothalamic PSA-NCAM is sufficient to alter energy homeostasis and promote fat storage under hypercaloric pressure, inter-individual variability in hypothalamic PSA-NCAM might account for the vulnerability to diet-induced obesity. These data support the concept that reduced plasticity in brain circuits that control appetite, metabolism and body weight confers risk for eating disorders and obesity.

Keywords: food intake, obesity, maladaptive eating behavior, synaptic plasticity, PSA-NCAM, polysialylation, brain, hypothalamus

INTRODUCTION

Changes in lifestyle and in the availability and quality of food largely explain the worldwide epidemic of obesity (1). However, high variability exists in individual susceptibility to develop overweight in an obesogenic environment (2). This implies that genetic risk factors among individuals substantially influence the variability in body mass index (3). A better understanding of the molecular basis of this heterogeneity might help to fight against obesity and its related disorders. In the past, numerous animal models have been used to investigate pre-existing differences that are established before the onset of corpulence and that confer a risk for common obesity (4–7). In this way, prospective studies in rats have shown that some early metabolic responses to high fat diet (HFD) at normal weight are predictive of propensity to develop obesity and liver diseases on the long term (8–11). In the present study, we found in mice that aberrant feeding behavior in response to dietary fat constitutes a latent vulnerability trait to obesity. This predictive model can be relevant to identify biological risk factors for maladpative eating behaviors and obesity before the onset of the disease. Recent genetic studies in human indicated that most of the genes associated to the body mass index are enriched in the brain and related to neuronal biology and synaptic plasticity (12, 13). Thus, we further examined the expression of polysialic acid-neural cell adhesion molecule (PSA-NCAM), a marker of neural plasticity, in mice prone to obesity, and we found that the vulnerability to obesity was correlated with decreased hypothalamus level of PSA-NCAM.

METHODS

Animals

Protocols including manipulation of animals (registration number 853.01) were reviewed and approved by our local Institutional Animal Care and Use Committee ("Comité d'éthique en expérimentation animale n°105"), and were in strict accordance with the European Community guidelines (directive 86/906). Experiments were carried out with 2 months-old male C57Bl/6JOla mice from Harlan Laboratories. Mice were housed individually, fed either a standard diet (catalog number A04; Safe Laboratories; Augy, France; 3.3 kcal/kg; energy from carbohydrate/fat/protein: 72.4/8.4/19.3) or a customized highly palatable high-fat diet (catalog number U8954P V.7; Safe Laboratories; 4.4 kcal/kg; energy from carbohydrate/fat/protein: 40.8/42.9/16.3). Free access was given for food and water. Recordings of food intake and body weight were done manually at 9:00 h. For tissue collection, mice were killed between 14:00 and 16:00 h after a 6-h fast.

Medio-Basal Hypothalamus Dissection

After sacrifice, brains were quickly removed and immersed in cold PBS solution. A 2 mm coronal slice was cut with a brain matrix. The slice was laid on a 6% agarose block and the MBH was dissected under stereomicroscope and cold-light illumination using a scalpel. Tissues were immediately frozen in liquid nitrogen and stored at −80°C until experiments.

PSA-NCAM Assay

Tissues were lysed and homogenized in RIPA lysis buffer using the TissueLyser system (Qiagen; Courtaboeuf, France) and 5 mm stainless steel beads (Qiagen). The homogenates were centrifuged 5 min at 5,000 *g* and supernatants were collected for PSA-NCAM assay using an enzyme-linked immunosorbent assay kit (PSA NCAM ELISA kit; Eurobio; Courtaboeuf, France) and for protein assay (Bio-Rad Protein Assay Kit ll; Biorad; Marnes-la-Coquette, France).

Statistical Analysis

All data are expressed as means. Error bars indicate standard errors of the mean (SEM). Multiple comparisons between groups were carried out by one- or two-way ANOVA using Prism 5.0 software (GraphPad Software; San Diego, CA, United States). *Post-hoc* analyses were done when main effects reached significance. Before comparison, Bartlett's and Shapiro–Wilk's tests were applied to check equality of variances and to evaluate the normality of the distribution, respectively.

RESULTS

The Homeostatic Feeding Response to a Dietary Fat Challenge Reveals Obesity Susceptibility

For a 2-month old mice fed with standard diet, the typical daily energy intake is 0.5 kcal per gram of body weight (The Jackson Laboratory, Mouse Phenome Database, http://phenome.jax.org/). It is well-known that high-energy foods cause transient overeating in most of the mice, which corresponds to an acute increase in energy intake during a few days (14–16), as illustrated in **Figure S1A**. In this study, the period of overconsumption was variable between mice and normalization of energy intake occurred after 2 days or more (**Figure S1B**; in kcal/gram of body weight). Consequently, the individual cumulative energy intake during 1-week HFD, hereinafter referred to as the "feeding response," ranged from 3.96 to 6.92 kcal/g (**Figure S1C**). According to this response, we identified by a median split HFD-intolerant mice with high feeding response due to slow normalization, and HFD-tolerant mice with low feeding response due to fast normalization (**Figures 1A,B**). The two groups of mice were initially undistinguishable with respect to their energy intake on standard diet or to their initial body weight (**Figures 1C,D**). On HFD, tolerant mice normalized their energy intake in only 2 days after HFD introduction, whereas intolerant mice normalized it after 10 days (**Figure 1E**; in kcal/gram of body weight). After 2 weeks on HFD, energy intake was normalized for all mice. Nevertheless, intolerant mice kept on HFD for a long term had further episodic increases in raw energy intake which appeared after 5 weeks on HFD (**Figure 1E**; in kcal). Therefore, the cumulative energy intake over 3 months with HFD was significantly higher in intolerant mice than in tolerant mice (**Figure 1F**). Moreover, the longitudinal follow-up showed that body weight of intolerant mice maintained on HFD increased strongly after 8 weeks in comparison to tolerant mice (**Figure 1G**). Hence, the body weight gain between the two groups of mice was significantly different after 3 months on HFD (**Figure 1H**). Importantly, the terminal body weight gain was positively correlated with the feeding response to HFD (**Figure 1I**). By contrast, it was not correlated with the initial energy intake on standard diet or with the terminal energy intake on HFD (**Figures 1J,K**). These results indicated that the homeostatic feeding response to an acute HFD challenge in mice is a latent trait predictive of the propensity to gain weight under persistent caloric pressure.

Relation Between Fat Tolerance and Hypothalamus Polysialylation

Identification of individuals prone to obesity before the onset of the disease allows investigation of the biological underpinnings of susceptibility to obesity and makes possible the discovery of biological risk factors. Pre-existing differences in brain circuits controlling appetite and energy homeostasis might account for the vulnerability to common obesity (17, 18). Recent genetic studies formally implicated the brain in obesity pathology and pinpointed neuronal genes regulating synaptic plasticity (12, 19, 20). Moreover, rodent data consistently evidenced alterations of neural function and plasticity in brain feeding circuits in animals genetically predisposed to obesity (21, 22). Thus, we hypothesized that innate alteration in molecules involved in brain neuroplasticity would confer risk for obesity.

The polysialic acid-neural cell adhesion molecule (PSA-NCAM) is a cell-surface molecule that promotes various plasticity-related changes in brain circuits (23). We recently identified PSA-NCAM in the hypothalamus as a permissive

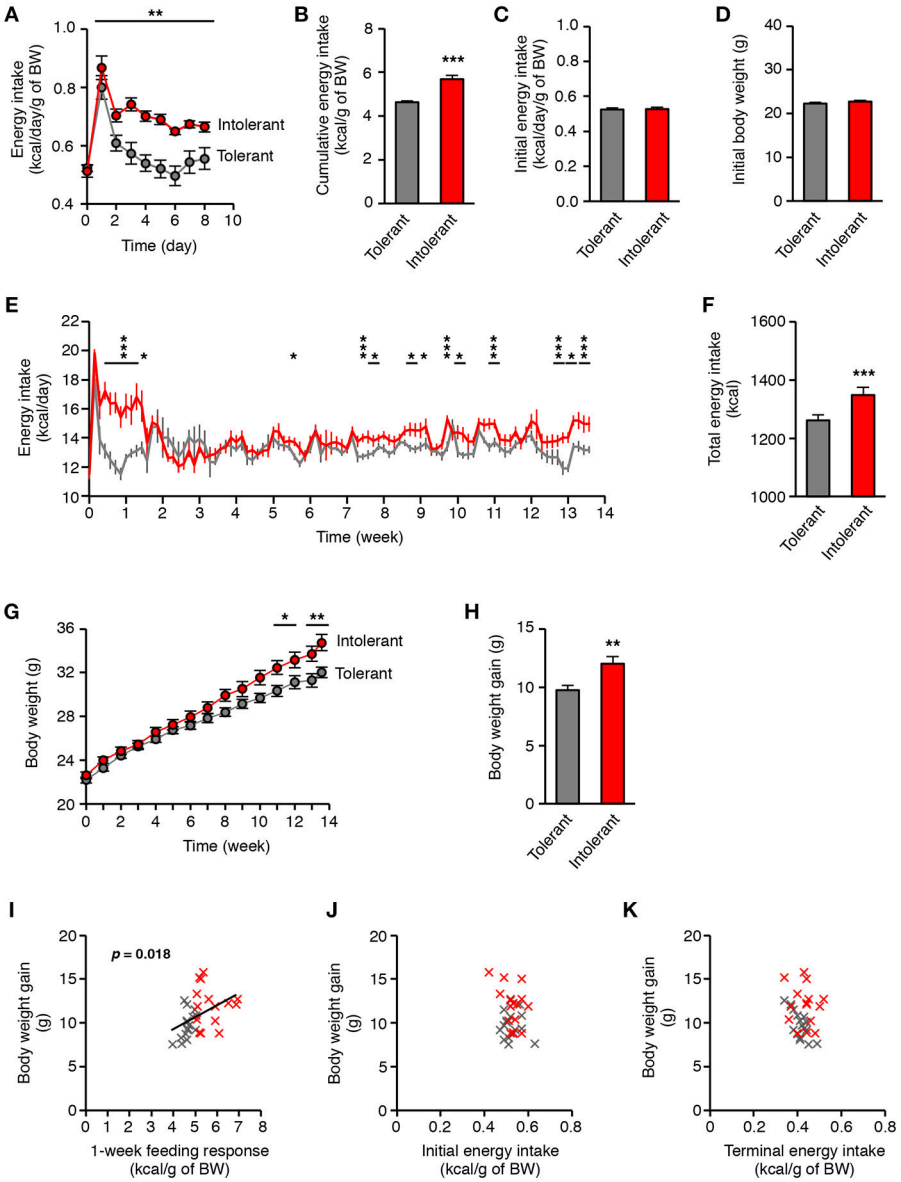

FIGURE 1 | Individual differences in the homeostatic feeding response to HFD reveal susceptibility to diet-induced obesity. **(A)** Energy intake per gram of body weight of HFD-tolerant ($n = 16$) and HFD-intolerant ($n = 15$) mice during 1-week HFD (**$p < 0.01$, HFD-tolerant vs. HFD-intolerant mice; two-way ANOVA and Bonferroni *post-hoc* test). **(B)** Cumulative energy intake per gram of body weight of HFD-tolerant and HFD-intolerant mice after 1 week on HFD (***$p < 0.001$; Mann Whitney test). **(C,D)** Initial energy intake per gram of body weight and body weight of HFD-tolerant and HFD-intolerant mice were similar on standard chow, i.e., before HFD introduction. **(E)** Energy intake of HFD-tolerant and HFD-intolerant mice during 13 weeks-HFD (*$p < 0.05$, ***$p < 0.001$, HFD-tolerant vs. HFD-intolerant mice; two-way ANOVA and Bonferroni *post-hoc* test). **(F)** Total energy intake of HFD-tolerant and HFD-intolerant mice after 3-month HFD (***$p < 0.001$; unpaired *t*-test). **(G)** Body weight of HFD-tolerant and HFD-intolerant mice during 13-week HFD (*$p < 0.05$, **$p < 0.01$, HFD-tolerant vs. HFD-intolerant mice; two-way ANOVA and Bonferroni *post-hoc* test). **(H)** Body weight gain of HFD-tolerant and HFD-intolerant mice after 13-week HFD (**$p < 0.01$; unpaired *t*-test). **(I)** Correlation analysis between the acute feeding response to HFD and the body weight gain after 3-month HFD. HFD-tolerant mice are in gray, HFD-intolerant mice are in red ($p < 0.018$; linear regression). **(J)** Correlation analysis between the initial energy intake on standard diet and the body weight gain after 3-month HFD. **(K)** Correlation analysis between the terminal energy intake on HFD and the body weight gain after 3-month HFD. All results are mean ± SEM.

factor for the brain control of energy balance (16, 24, 25). We therefore postulated that low level of hypothalamic PSA-NCAM would incite vulnerability to obesity. To test this hypothesis, we identified in a second cohort of mice ($n = 14$), HFD-tolerant and HFD-intolerant individuals according to their feeding response to 1-week HFD and then we examined the concentration of PSA-NCAM in the medio-basal hypothalamus (MBH) collected after this oral fat tolerance test. Intolerant mice showed significant lower levels of hypothalamic PSA-NCAM in comparison to tolerant mice (**Figure 2**; tolerant: 684.7 ± 59.4 ng/mg; intolerant:

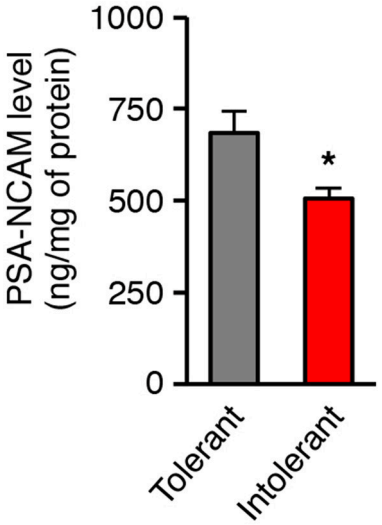

FIGURE 2 | Individual differences in the tolerance to HFD are linked to the level of hypothalamic PSA-NCAM. PSA-NCAM levels were measured in mediobasal hypothalamus after a 1-week HFD challenge allowing identification of HFD-tolerant and HFD-intolerant mice ($n = 7$ HFD-tolerant, $n = 7$ HFD-intolerant; $^*p < 0.05$; Mann Whitney test). Results are mean ± SEM.

505.9 ± 27.1 ng/mg; $p = 0.038$). As a reference, the constitutive level of PSA-NCAM in the MBH of control mice fed a standard diet was 508.5 ± 31 ng/mg protein ($n = 16$). The level of PSA-NCAM in the MBH of intolerant mice did not differ from the constitutive value. These data suggest that tolerant mice might have higher ability to mobilize PSA-NCAM to trigger synaptic plasticity in the hypothalamus than intolerant mice.

DISCUSSION

In this study, we measured the feeding response to 1-week HFD in mice. We found variability in the behavioral response with high responders who failed to rapidly normalize their energy intake during the metabolic challenge. High responders also gained more weight when they consumed the hypercaloric diet for a long time. Thus, the 1-week HFD challenge may be considered as a Dietary Fat Tolerance Test (DFTT) for identifying mice prone to nutritional obesity. Similar predictive tests have been used in the past showing that short-term response to HFD constitutes a phenotypic trait of metabolic flexibility which provides information on vulnerability to metabolic diseases (8–11). The present study further shows that the DFTT could be based on the behavioral response only, and does not necessarily imply collecting biopsies for metabolic investigation.

The DFTT might be relevant for investigating intrinsic factors that produce maladaptive eating disorders and vulnerability to obesity before the clinical onset of the disease. Some of these factors have been already evidenced, including efficiency of peripheral endocrinological and metabolic processes (8, 9, 26, 27), and responsivness of the dopaminergic system to food cues (28). Here we show that interindividual variability in the feeding response to short-term HFD is also linked to the intrahypothalamic PSA-NCAM signaling. This is in line with previous works from our group showing that experimental removal of hypothalamic PSA-NCAM increases the feeding response to HFD and the body weight gain (16, 24).

After the DFTT, intrahypothalamic PSA-NCAM content was elevated in HFD-tolerant mice, while it remained low and similar to the constitutive level in HFD-intolerant mice. This result suggests that inter-individual variability exists in the ability to upregulate PSA-NCAM production in the hypothalamus in response to the metabolic challenge, and that PSA-NCAM production was stimulated in HFD-tolerant mice. The source of variation of PSA-NCAM expression between HFD-tolerant and -intolerant animals is not known. Similar intrinsic differences in healthy animals have been already reported, linking the level of prefrontal PSA-NCAM to the susceptibility for addiction-related behaviors (29). Interindividual differences in learning-induced PSA-NCAM levels in the hippocampus is also linked to learning ability in rats (30). Interestingly, acute stress that predisposes young rats to mood and anxiety disorders during adulthood, as well as chronic stress in adult, cause dramatic alteration in PSA-NCAM expression in the limbic system (31, 32). These results show that brain PSA-NCAM synthesis is affected not only by genetic heritage but also by experience. Since litter size and maternal care are early determinants of vulnerability to obesity (33–36), PSA-NCAM variation in our model might by linked to perinatal experiences. At the molecular level, biosynthesis of PSA-NCAM in adult brain requires successive biochemical reactions, which are catalyzed by enzymes including UDP-GlcNAc 2-epimerase/ManNAc kinase and polysialyltransferase 1, encoded by *Gne* and *St8sia4* genes, respectively. One can propose that these genes might be differentially upregulated on HFD depending on individuals. Gene polymorphism association studies and epigenetic research on these specific genes in murine models and in humans are thus promising research to increase our knowledge on obesity susceptibility.

In several models of neuropsychological disorders, the loss of neural PSA-NCAM alters physiological defenses, exacerbates symptoms or worsens the disease (29, 37–40), pointing out the potential protective role of PSA-NCAM against brain dysfunctions involving a plasticity-related component. In previous works, we found that removal of hypothalamic PSA is sufficient to alter short-term homeostatic responses to dietary fat and increase body weight gain on a long term (16, 24, 25), confirming that hypothalamic PSA-NCAM is critical to maintain energy homeostasis upon metabolic challenge. Together, these data suggest a causal link between hypothalamic PSA-NCAM and the propensity for obesity. Given the role of PSA-NCAM in brain plasticity (23, 41), our findings further strengthen the concept that factors controlling neural plasticity are critical in the individual susceptibility to develop overweight with obesogenic foods (22, 42, 43). Consistently, a growing body of evidence indicates that weight gain in human is associated to genes encoding plasticity-related molecules (12, 19, 20, 44–47).

Future research is now needed to understand molecular basis of inter-individual heterogeneity in hypothalamic PSA-NCAM level and to identify life stressors that influence the expression of this factor in the hypothalamus.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2018.00125/full#supplementary-material

Figure S1 | Acute feeding response of HFD-fed mice. **(A)** Average homeostatic feeding response to HFD in 2-month old male mice (C57Bl/6JOla) ($n = 31$; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; one-way ANOVA for repeated measures followed by multiple comparison Dunnett *post-hoc* test vs. day 0). **(B)** Graphical representation of the individual feeding responses to 1-week HFD in 2-month old male mice (C57Bl/6JOla) ($n = 31$). **(C)** Plotting of the cumulative energy intake of mice on HFD for 1 week. Median split separated HFD-tolerant mice with low feeding response (gray; $n = 16$) and HFD-intolerant mice with high feeding response (red; $n = 15$).

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The *anx/anx* Mouse – A Valuable Resource in Anorexia Nervosa Research

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Animal models are invaluable resources in research concerning the neurobiology of anorexia nervosa (AN), to a large extent since valid clinical samples are rare. None of the existing models can capture all aspects of AN but they are able to mirror the core features of the disorder e.g., elective starvation, emaciation and premature death. The anorectic *anx/anx* mouse is of particular value for the understanding of the abnormal response to negative energy balance seen in AN. These mice appear normal at birth but gradually develops starvation and emaciation despite full access to food, and die prematurely around three weeks of age. Several changes in hypothalamic neuropeptidergic and -transmitter systems involved in regulating food intake and metabolism have been documented in the *anx/anx* mouse. These changes are accompanied by signs of inflammation and degeneration in the same hypothalamic regions; including activation of microglia cells and expression of major histocompatibility complex I by microglia and selective neuronal populations. These aberrances are likely related to the dysfunction of complex I (CI) in the oxidative phosphorylation system of the mitochondria, and subsequent increased oxidative stress, which also has been revealed in the hypothalamus of these mice. Interestingly, a similar CI dysfunction has been shown in leukocytes from patients with AN. In addition, a higher expression of the *Neurotrophic Receptor Tyrosine Kinase 3* gene has been shown in the *anx/anx* hypothalamus. This agrees with AN being associated with specific variants of the genes for brain derived neurotrophic factor and Neurotrophic Receptor Tyrosine Kinase 2. The *anx/anx* mouse is also glucose intolerant and display pancreatic dysfunction related to increased levels of circulating free fatty acids (FFA) and pancreatic inflammation. An increased incidence of eating disorders has been reported for young diabetic women, and as well has increased levels of circulating FFAs in AN. Also similar to individuals with AN, the *anx/anx* mouse has reduced leptin and increased cholesterol levels in serum. Thus, the *anx/anx* mouse shares several characteristics with patients with AN, including emaciation, starvation, premature death, diabetic features, increased FFA and low leptin, and is therefore a unique resource in research on the (neuro)biology of AN.

Keywords: hypothalamus, anorexia, inflammation, neurodegeneration, neuropeptide, AGRP, microglia

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INTRODUCTION – ANOREXIA NERVOSA

Anorexia nervosa (AN) is a complex psychiatric disorder affecting around 1% of females and 0.1% of males, of which as many as 10% die as a result of the disorder (Bulik et al., 2006; Keski-Rahkonen et al., 2007; Papadopoulos et al., 2009). The diagnostic criteria, according to the Diagnostic and statistical manual of mental disorders (DSMV), include persistent food intake restriction leading to significantly low body weight, combined with persistent behaviors that interfere with weight gain, and body image distortion (Schaumberg et al., 2017). One central and yet unexplained part of AN is the contradictory response to negative energy balance and the inability to ingest adequate energy, leading to severe underweight. It is indeed paradoxical that while most individuals quickly regain the weight lost from dieting (Pietilainen et al., 2012), individuals with AN stay in an emaciated state commonly for many years, some even until death. It has been speculated that hunger signals are diminished or even absent in individuals with AN, and that satiety signals on the other hand are exaggerated (DeBoer, 2011; Oberndorfer et al., 2013). Supporting this hypothesis, a genome wide association study (GWAS), as well as genetic correlation data, indicate that individuals with AN are genetically predisposed to a lower body weight set point (Duncan et al., 2017; Hinney et al., 2017). However, in order to understand the complex biology of AN, in particular the illogical response to starvation and underweight, we need to learn more about the neurobiological pathways and molecular mechanisms that are associated with severe dysregulation of food intake. This is something that is technically difficult and to some extent impossible to do in humans, since post-mortem tissues rarely are available. On the other hand, animal models cannot capture all aspects of AN but they are able to mirror the core features of the disorder e.g., elective starvation, emaciation and premature death (Siegfried et al., 2003). Animal models have therefore proved to be invaluable resources for researchers in the field. One such model is the *anx/anx* mouse.

THE *ANX/ANX* MOUSE

The homozygous *anx*-mouse appears normal at birth, meaning that it is indistinguishable from their homozygous and heterozygous wildtype (wt) siblings. However, during the first postnatal weeks they gradually develop the core symptoms of AN; starvation and emaciation (**Figure 1**). The *anx/anx* mouse dies prematurely around 3 weeks of age, and by then weigh around half as much as their siblings. They are able to eat, but despite full access to milk from the mother, eat significantly less already from postnatal day (P) 5. Worth to note is that the diurnal patterns in food intake seen in their healthy siblings are mirrored in the *anx/anx* mouse, even though the amount ingested is significantly smaller (Maltais et al., 1984). Neurological/behavioral deviations such as head weaving, hyperactivity, body tremors and uncoordinated gait, were described in the original paper by Maltais et al. (1984). When corrected for body weight, brain and thymus weights are increased compared to their healthy siblings, both at P5 and P15, while the weight of spleen is reduced (Maltais et al., 1984). See **Table 1** for a summary of the aberrances in the *anx/anx* mouse discussed here and below.

The *anx* Mutation

The *anx* mutation arose spontaneously at the Jackson laboratory in Bar Harbor, Maine, already in 1976 in the F2 generation of a cross between DW/J and an inbred strain, the latter was derived from a cross between M.m.poschiavinus and an inbred Swiss strain. The male *anx* carrier was crossed to a female B6C3H-a/a F1 mouse, and the mutation has since then been conserved on this background (Maltais et al., 1984). We have mapped the mutation to a 0.2 cM interval residing between the markers D2Mit133 and Jojo5 chromosome 2 (Chr 2: bp 118, 889, 896–120, 175, 108[1]) (Lindfors et al., 2011). So far, no sequencing attempts have been able to show any unique sequence alteration. However, one needs to keep in mind that the background of the *anx/anx* mouse includes five different strains (see above) which makes *de novo* assembly difficult. The lack of unique finding could also mean that the mutation is located in a regulatory element outside the interval. The NADH dehydrogenase (ubiquinone) 1a-subcomplex (*Ndufaf1*) gene, shown to be closely associated with several of the *anx/anx* phenotypes, is however, located in the short interval of the mutation (see section on mitochondrial dysfunction below) (Lindfors et al., 2011). *Ndufaf1* is an assembly factor for complex I (CI) in the mitochondrial oxidative phosphorylation system (OXPHOS) (Vogel et al., 2005). In addition, work by Kim et al. (2017) identified a point mutation in *Tyro3* which they conclude is not the *anx*-mutation but a strain specific modifier of *anx*-phenotypes (Kim et al., 2017). Thus, despite that the *anx/anx* mouse model recently turned 40 years, the mutation is still unknown. Hopefully modern techniques within e.g., sequencing will be able to shed light on this mystery.

Neurochemistry of the *anx/anx* Mouse

Several changes in neuropeptidergic and -transmitter systems, in particular systems in the hypothalamus known to regulate food intake and metabolism (energy homeostasis), have been documented in the *anx/anx* brain (Broberger et al., 1997, 1999; Johansen et al., 2000, 2003; Nilsson et al., 2013). A part of the hypothalamus, called the Arcuate nucleus (Arc), is of particular importance concerning energy homeostasis. The Arc harbors among others a neuronal population co-expressing two orexigenic neuropeptides; agouti-gene related protein (AGRP) and neuropeptide Y (NPY), and a neuronal population co-expressing the anorexigenic peptide/precursor; pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) (Chronwall, 1985; Cone et al., 2001; Schwartz, 2001). Aberrances have been documented in both these neuronal populations in the *anx/anx* mouse. Immunohistochemistry revealed increased number of NPY and AGRP immunopositive cell bodies within the Arc, combined

[1] www.ensembl.org

FIGURE 1 | An *anx/anx* mouse and wildtype (+/+) littermate, age 17 days.

with a reduction in AGRP/NPY immunopositive projections in the hypothalamic and extra-hypothalamic target areas of these neurons (Broberger et al., 1997, 1998; Fetissov et al., 2005; Nilsson et al., 2008). *In situ* hybridization studies have with regard to these neuropeptides been inconsistent, which most likely is attributed to overexposure of the labeled glass slides in the earlier studies. Thus, while initial studies documented no change in mRNA levels of NPY in the Arc of the *anx/anx* mouse (Broberger et al., 1997; Jahng et al., 1998), a later study showed increased mRNA for both NPY and AGRP in the *anx/anx* Arc (Fetissov et al., 2005). With regard to the POMC/CART population, significantly decreased levels of CART mRNA, as well as CART immunopositive cell bodies and fibers in Arc have been shown in the *anx/anx* hypothalamus. Also, a lower number of detectable CART-expressing cells in the dorsomedial hypothalamic nucleus/lateral hypothalamic area is seen (Johansen et al., 2000). *In situ* hybridization demonstrated decreased numbers of POMC-expressing neurons in the *anx/anx* Arc (Broberger et al., 1999). Using the neuropeptide Y receptor 1 (Y1) which outlines the soma and dendrites of POMC/CART neurons (Zhang et al., 1994; Kopp et al., 2002), markedly reduced immunoreactivity in Arc and the paraventricular nucleus of hypothalamus was revealed (Broberger et al., 1999; Nilsson et al., 2011). Clinically, genetic variants of AGRP have been associated with AN (Dardennes et al., 2007) or with lowest BMI during AN illness (Yilmaz et al., 2014). Increased plasma levels of the peptide have been documented in AN (Moriya et al., 2006), but it is so far unknown if this change remains after weight recovery. The changed cerebrospinal fluid levels of NPY seen in AN is however, known to be secondary to the illness (Gendall et al., 1999).

In addition, an increased expression of the neurotrophic receptor kinase 3 (*Ntrk3)* gene has been shown in the *anx/anx* hypothalamus (Mercader et al., 2008b). This agrees with AN being associated with specific variants of the genes for brain derived neurotrophic factor (BDNF) and neurotrophic receptor tyrosine kinase 2 (NTRK2) (Ribases et al., 2003, 2005).

Changes have been documented also in other brain regions than the hypothalamus. Increased apoptosis and proliferation in the dentate gyrus of the hippocampus (Kim et al., 2001), serotonergic hyperinnervation in hippocampus, cortex, olfactory bulb and cerebellum (Son et al., 1994), as well as altered dopaminergic transmission in the striatum (Johansen et al., 2001), have been demonstrated. Genetic variants as well as deviant levels of metabolites and receptors related to dopamine and serotonin have been linked to the AN pathology (Kaye et al., 1999, 2005; Kaye, 2008).

TABLE 1 | Main characteristics of the *anx/anx* mouse.

Aberrances of the *anx/anx* mouse	Reference
Major phenotypes: reduced food intake, emaciation and premature death.	Maltais et al., 1984
Organ changes: increased weight of thymus and brain, and reduced weight of spleen.	Maltais et al., 1984
Behavioral/Neurological phenotypes: head weaving, tremor, hyperactivity and uncoordinated gait.	Maltais et al., 1984
Hypothalamic neuropeptidergic/-transmitter and molecular aberrances: - *AGRP/NPY*: increased number of AGRP/NPY-immunopositive cell bodies in Arc, reduced number of immunopositive projections. N.C /reduced mRNA expression of AGRP and NPY in Arc. - *POMC/CART*: Reduced number of CART-immunopositive cell bodies in Arc, DMH, LHA, reduced number of immunopositive projections in Arc. Reduced number of Y1-immunopositive cell bodies and projections. Reduced POMC mRNA in Arc. - Increased hypothalamic expression of *Ntrk3*.	Broberger et al., 1998; Nilsson et al., 2008 Jahng et al., 1998; Fetissov et al., 2005 Johansen et al., 2000 Broberger et al., 1999; Nilsson et al., 2011 Mercader et al., 2008b
Hypothalamic inflammation, e.g., microglia activation and expression of MHC class I by hypothalamic microglia.	Lachuer et al., 2005; Mercader et al., 2008a; Nilsson et al., 2008
Hypothalamic degeneration, e.g., expression of MHC class I by Arc neurons, microglia-associated cell death, increased TUNEL labeling in Arc.	Nilsson et al., 2011
Mitochondrial dysfunction, e.g., down regulation of *Ndufaf1* and reduced capacity of CI.	Lindfors et al., 2011
Neurotransmitter changes in other parts of the brain: - Increased apoptosis and proliferation in hippocampus. - Serotonergic hyperinnervation of hippocampus, striatum, cortex and cerebellum. - Altered dopaminergic neurotransmission.	Kim et al., 2001 Son et al., 1994 Johansen et al., 2001
Pancreatic aberrances, e.g., glucose intolerance, reduced insulin release and inflammation.	Lindfors et al., 2015
Reduced hypothalamic metabolism, e.g., reduced glucose uptake, lactate and activation of AMPK, and increased PCr.	Bergstrom et al., 2017
Changes in serum metabolites, i.e., reduced leptin and increased FFA.	Johansen et al., 2000; Lindfors et al., 2015

AGRP, agouti-gene related protein; AMPK, AMP-activated kinase; Arc, the Arcuate nucleus; CI, complex I of the oxidative phosphorylation system; CART, cocaine and amphetamine-regulated transcript; DMH, the dorsomedial hypothalamic nucleus; FFA, free fatty acids; LHA, the lateral hypothalamic area; MHC class I, major histocompatibility complex I; N.C, no change; Ndufaf1, The NADH dehydrogenase (ubiquinone) 1a-subcomplex gene; NPY, neuropeptide Y; Ntrk3, neurotrophic receptor kinase 3 gene; PCr, phosphocreatine; POMC, pro-opiomelanocortin; TUNEL, terminal dUTP nick end labeling; Y1, neuropeptide Y receptor 1.

Neuroinflammation and Degeneration in the anx/anx Hypothalamus

The hypothalamic neurochemical aberrances of the *anx/anx* mouse are accompanied by signs of inflammation and degeneration (Lachuer et al., 2005; Mercader et al., 2008a;

Nilsson et al., 2008, 2011). Microglia cells are immunocompetent cells that are activated in the central nervous system in response to e.g., inflammation, neurodegeneration or injury (Nakajima and Kohsaka, 2004; Streit et al., 2005). In the *anx/anx* brain, microglia are activated selectively in the hypothalamic regions where the neurons, both cell bodies and projections, expressing the orexigenic neuropeptide AGRP are located (Nilsson et al., 2008). The first appearance of activated microglia overlaps in time with the loss of AGRP immunoreactive projections, i.e., P12–15 (Nilsson et al., 2008). Similarly, chemical ablation of the AGRP neurons results in starvation in both normal weight and obese mice, and results in glia (microglia and astroglia) activation in the target areas (Wu et al., 2008, 2012). Major histocompatibility complex I is expressed by the activated microglia, but also by the AGRP and POMC expressing neurons in the *anx/anx* brain (Nilsson et al., 2011). This latter finding combined with increased hypothalamic terminal dUTP nick end labeling (TUNEL) labeling and so called microglia-associated cell death (Ribak et al., 2009), made us conclude that hypothalamic degeneration is associated with the anorexia of the *anx/anx* mouse (Nilsson et al., 2011). In addition, two microarray studies of the *anx/anx* hypothalamus revealed changed expression of an enrichment of genes involved in inflammation and cell death (Lachuer et al., 2005; Mercader et al., 2008a). While it is unknown if hypothalamic inflammation occurs in AN, it has been linked to cachexia, the anorexia that often accompanies chronic illnesses such as cancer and HIV (Durham et al., 2009; Dwarkasing et al., 2016).

Mitochondrial CI Dysfunction and Reduced Hypothalamic Metabolism

A dysfunction selective of CI in OXPHOS, and subsequent increased oxidative stress, have been revealed in the hypothalamus of the *anx/anx* mouse (Lindfors et al., 2011). This CI dysfunction is connected to down regulation of the gene *Ndufaf1* which in fact is located in the *anx* interval (see section on the *anx* mutation above). The down regulation has been confirmed at the protein level at P21 (Lindfors et al., 2011). *Ndufaf1* encodes one of several proteins crucial for the correct assembly of the 44–46 proteins that build up CI (Smeitink et al., 2001; Ugalde et al., 2004a,b; Guerrero-Castillo et al., 2017). Selective neuronal damage and glia activation, as shown in the *anx/anx* mouse (Nilsson et al., 2008, 2011), has been shown in another animal model with CI deficiencies, i.e., the Ndufs4-KO mouse (Quintana et al., 2010). The NDUFAF1 gene, as well as other players in CI biogenesis, have been implicated in human pathology; resulting in e.g., leukodystrophy and failure to thrive in young children (Vogel et al., 2005, 2007; Dunning et al., 2007; Distelmaier et al., 2009). In fact, CI dysfunction has been shown in leukocytes from patients with AN (Victor et al., 2014), but it remains to be explored if this is a cause or consequence of the disorder. This far, the NDUFAF1 gene has not been associated with AN, but it would be worth exploring genetics variants related to OXPHOS function and a potential association with AN, similar to what has been shown in other psychiatric disorders e.g., autism spectrum disorder (Giulivi et al., 2010). With this saying the *anx/anx* model is a model of value for research on all human conditions with loss of appetite i.e., anorexia, including the anorexia seen in cachexia and failure to thrive, as well as AN. The *anx/anx* mouse is unique in the sense that few other models exist were the mice, similarly to the human conditions just mentioned, eat insufficient despite having full access to food. This in contrast to models were the researcher in one way or another limits the access of food (Siegfried et al., 2003).

Diseases associated with mitochondrial dysfunction are commonly associated with a stressed metabolic profile, and hypermetabolism (Wredenberg et al., 2006; Jeppesen et al., 2007; Milone and Wong, 2013). Supposedly such metabolic responses occur in order to safeguard adequate levels of ATP. In some cases, conversely, mitochondrial dysfunction is associated with reduced glucose uptake and hypometabolism, e.g., in Alzheimer's disease and epilepsy (Chandrasekaran et al., 1996; Tenney et al., 2014). This resembles what we saw in the *anx/anx* hypothalamus, i.e., lower glucose uptake rate, decreased lactate content, as well as elevated phosphocreatine (PCr) content and reduced activation of AMP-activated kinase (AMPK) in the basal state (Bergstrom et al., 2017). This is similar to the hypometabolic state seen in hibernation (Healy et al., 2011) and could be reflecting lower neuronal activity (Cunnane et al., 2011). Different neuronal populations respond differently to this type of metabolic stress (Schreiber and Baudry, 1995), which has been ascribed to the subtype of ATP-sensitive potassium channel (K-ATP) they express. A specific subtype of K-ATP channel that consists of Kir 6.2 and SUR1 subunits becomes activated by mitochondrial CI dysfunction, i.e., by increased ROS levels and/or reduced levels of ATP. This leads to ceased electrical activity, hyperpolarization and reduced firing, in a means of protecting the cell from the energy deficiency and increased oxidative stress (Liss et al., 1999). Kir6.2/SUR1 K-ATP channels are expressed by the hypothalamic POMC/CART and AGRP/NPY neurons, and by a limited number of other cell populations including the pancreatic beta-cells and dopaminergic neurons in Substantia Nigra (Miki et al., 2001; Ibrahim et al., 2003; van den Top and Spanswick, 2006; van den Top et al., 2007). Firing of action potentials and release of neurotransmitters are processes that require high amounts of energy. Therefore, inhibition of these processes would conserve energy during conditions when energy is scarce (Attwell and Laughlin, 2001; Sengupta et al., 2010). In addition, uncontrolled generation of ROS, commonly accompanying CI dysfunction, can also cause diminished firing of the AGRP/NPY neurons, thus resulting in a reduced orexigenic drive (Andrews et al., 2008; Horvath et al., 2009).

Pancreatic Dysfunction and Aberrant Levels of Fat Derived Molecules

The *anx/anx* mouse also displays a pancreatic dysfunction (Lindfors et al., 2015). More specifically, they are markedly glucose intolerant, and show reduced insulin release upon glucose tolerance test. This is associated with elevated serum concentrations of free fatty acids (FFAs) in the *anx/anx* mouse and increased macrophage infiltration [indicative of inflammation (Imai et al., 1996; Ka et al., 2015)] of *anx/anx*

islets. Increased levels of FFAs have been connected to inhibition of glucose-induced insulin secretion (Eguchi et al., 2012). Interestingly, isolated *anx/anx* islets cultured in the absence of FFAs show increased insulin release upon glucose stimulation and show no signs of inflammation. Thus, the diabetic phenotype of the *anx/anx* mouse seems to be related to the elevated FFAs and inflammation in pancreatic islets. This finding is interesting in the light of the increased incidence of eating disorders that has been reported in young women with diabetes (Hudson et al., 1985; Meltzer et al., 2001), and documented increased levels of circulating FFAs in AN (Pinter et al., 1975; Curatola et al., 2004). Also similar to individuals with AN, the *anx/anx* mouse has low levels of the fat derived and food intake regulating hormone leptin, and high levels of cholesterol in serum (Maltais et al., 1984; Schorr and Miller, 2017).

CONCLUSION AND FUTURE PERSPECTIVE

The *anx/anx* mouse shares several characteristics with patients with AN, including emaciation, starvation, premature death, diabetic features, increased FFA and low leptin, and is therefore a unique and very valuable resource in order to explore and understand the (neuro)biology of AN. Future research should explore if hypothalamic inflammation and/or degeneration, as seen in the *anx/anx* mouse, are mechanisms involved also in AN. Further studies are also needed in order to clarify if the mitochondrial dysfunction seen in AN (Victor et al., 2014) is a cause or consequence of the disorder. Finally, it would be of value to be able to define the *anx*-mutation, as well as explore other brain areas related to food intake regulation, e.g., nucleus tractus solitarius and the parabrachial nucleus in the *anx/anx* mouse.

AUTHOR CONTRIBUTIONS

IN reviewed the literature, wrote, and edited the manuscript.

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Inter-individual Variability for High Fat Diet Consumption in Inbred C57BL/6 Mice

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Since inbred C57BL/6 mice are known to show inter-individual phenotypic variability for some traits, we tested the hypothesis that inbred C57BL/6 mice display a different tendency to consume a high fat (HF) diet. For this purpose, we used a compilation of HF intake data from an experimental protocol in which satiated mice were exposed to a HF pellet every morning for 2-h over 4 consecutive days. We found that mice displayed a large degree of variability in HF intake. Since day 1 HF intake significantly correlated with HF intake in successive days, we applied a hierarchical clustering algorithm on HF intake measurements in days 2, 3, and 4 in order to classify mice into "low" or "high" HF intake groups. "Low" HF intake group showed a day 1 HF intake similar to that seen in mice exposed to regular chow, while "high" HF intake group showed a higher day 1 HF intake as compared to "low" HF intake group. Both groups of mice increased HF consumption over the successive days, but "high" HF intake group always displayed a higher HF consumption than the "low" HF intake group. As compared to "low" HF intake group, "high" HF intake group showed a higher number of dopamine neurons positive for c-Fos in the VTA after the last event of HF intake. Thus, inbred C57BL/6 mice show inter-individual variability for HF intake and such feature may be linked to a different response to the rewarding properties of the HF diet.

Keywords: food intake, inter-individuality, palatable foods, eating behaviors, obesity

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INTRODUCTION

The worldwide obesity epidemic is recognized as one of the most serious global health problems. A key factor favoring body weight gain in humans is the modern lifestyle that constantly promotes the consumption of energy-dense foods. Thus, understanding the neurobiological systems that control the intake of some types of foods is essential for the development of novel strategies to tackle this growing problem. Most basic research concerning the neurobiological mechanisms controlling eating behaviors is performed in mice, which can be fed with a high-fat (HF) diet as a maneuver to mimic the increasing availability of palatable energy-dense foods in modern societies. Mice exposed to a HF diet increase their food intake, presumably because the rewarding aspects of eating override the homeostatic control of appetite and lead to the overconsumption of palatable stimuli independently of energy needs (1). Processing of rewarding aspects of food intake involve

the mesolimbic pathway that includes the dopamine neurons of the ventral tegmental area (VTA), which mainly innervate the nucleus accumbens and olfactory tubercle as well as other brain areas (2). Despite many important advances in the recent years, the mechanisms by which VTA dopamine neurons modulate behavioral functions related to food reward remain poorly known (3).

In order to investigate the neurobiology of HF intake, our group set up a simple experimental paradigm in which mice display a robust intake of HF diet. In particular, satiated mice are exposed to a HF pellet every morning for 2-h over 4 consecutive days, while maintaining free access to regular chow (RC). Given the experimental conditions, the rewarding aspects of appetite play a major role regulating HF intake. In line with this notion, HF intake under this experimental paradigm increases the expression of the marker of neuronal activation c-Fos in most centers of the mesolimbic pathway (4). After collecting food intake data from mice with daily and time-limited access to HF diet over several studies (4–6), it became evident that individual mice differed notably in their tendency to consume HF diet. The presence of such phenotypic variability was masked when data was averaged, and, as a consequence, this phenomenon was overlooked in our previous publications. Here, we present a compilation of HF intake data of inbred C57BL/6 wild type mice collected in our laboratory over the past years and present a more compelling analysis. In addition, we analyzed the potential association between the individual tendency of mice to consume a HF diet and the level of activation of the VTA dopamine neurons, as estimated by the induction of c-Fos expression.

METHODS

Animals

All studies were performed using naïve adult (2–6-month-old) mice from the inbred C57BL/6 strain that were generated in the animal facility of the IMBICE. Studies were performed using male mice in order to minimize the additional variability on eating behavior introduced by hormonal changes across ovulatory cycle. At weaning (21-day-old), mice were group-housed (6 mice/cage) and *ad libitum* fed with RC. Mice were housed under a 12-h light-dark cycle (lights switched on at 6:00 a.m.), at 22 ± 1°C room temperature. The study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimentation received approval from the Institutional Animal Care and Use Committee of the Multidisciplinary Institute of Cell Biology (approval IDs 10-0113 and 15-0122).

Diets

Diets were provided by Gepsa (Grupo Pilar, www.gepsa.com) and their color, texture and overall appearance were similar (7). **Table 1** shows the composition of RC and HF diet.

Experimental Protocol

Mice were single-housed in clean cages 3 days before the experiment and *ad libitum* fed with RC. For the experiments, mice were randomly assigned into: 1) a "HF group," which included mice daily exposed to a HF pellet inside their home cages from 9:00 to 11:00 a.m. during 4 consecutive days, or 2) a "RC group," which included mice daily exposed to a RC pellet in their home cages from 9:00 to 11:00 a.m. All mice remained with free access to RC in the hopper. RC and HF pellets offered to mice were pre-weighed and food intake was calculated by weighing all remnants of the pellet at 11:00 a.m. and subtracting it to the initial weight. Energy intake was calculated by multiplying food intake by the respective energy content of each diet. The fourth day after the experimental protocol, a set of mice from HF and RC groups ($n = 12$ and 7, respectively) was euthanized and perfused as previously described (8); their brains were extracted, frozen, coronally cut in four equivalent series in a cryostat and stored in cryopreservant solution at −20°C until processing.

TABLE 1 | Composition of the diets.

	RC	HF diet
Nutrient composition (g/kg)		
Protein	253	227
Total fat	36	210
Carbohydrate	500	400
Crude fiber	60	47
Ash	80	56
Moisture	71	60
Metabolizable energy (kcal/kg)	3,003	3,960

Ingredients: Corn, gluten meal, wheat middlings, soy flour, fish meal, meat meal, wheat, barley, ground oats, alfalfa meal, sunflower flour, chicken oil, vitamin mix, mineral mix. RC and HF diet contain 36 and 210 g/kg, respectively, of chicken oil added as a source of fat.

Immunostaining

One series of brain sections was used for c-Fos and tyrosine hydroxylase (TH) immunostaining, which was performed as described before (4). Briefly, sections were treated with 0.5% H_2O_2 and then treated with blocking solution (3% normal donkey serum and 0.25% Triton X-100). Next, sections were incubated with an anti-c-Fos antibody (Santa Cruz Biotechnology, cat# sc-7202, 1:2,000) for 48 h at 4°C. Then, sections were incubated with a biotinylated donkey anti-rabbit antibody (Vector Laboratories, cat# BA-1000, 1:1,000) and with reagents of the Vectastain Elite ABC kit (Vector Laboratories, cat# PK-6200), according to the manufacturer's protocols. Next, sections were incubated with 3-3′-diaminobencidine (DAB)/nickel solution in order to generate a black precipitate in c-Fos positive (c-Fos+) cells. Afterwards, sections were incubated with a rabbit anti-TH antibody (Santa Cruz, cat# sc-14007, 1:20,000) for 48 h at 4°C and sequentially incubated with a biotinylated donkey anti-rabbit antibody, reagents of the Vectastain Elite ABC kit and a DAB solution without nickel in order to generate a brown precipitate in the cytoplasm of TH positive (TH+) cells. Finally, sections were mounted and coverslipped with mounting media.

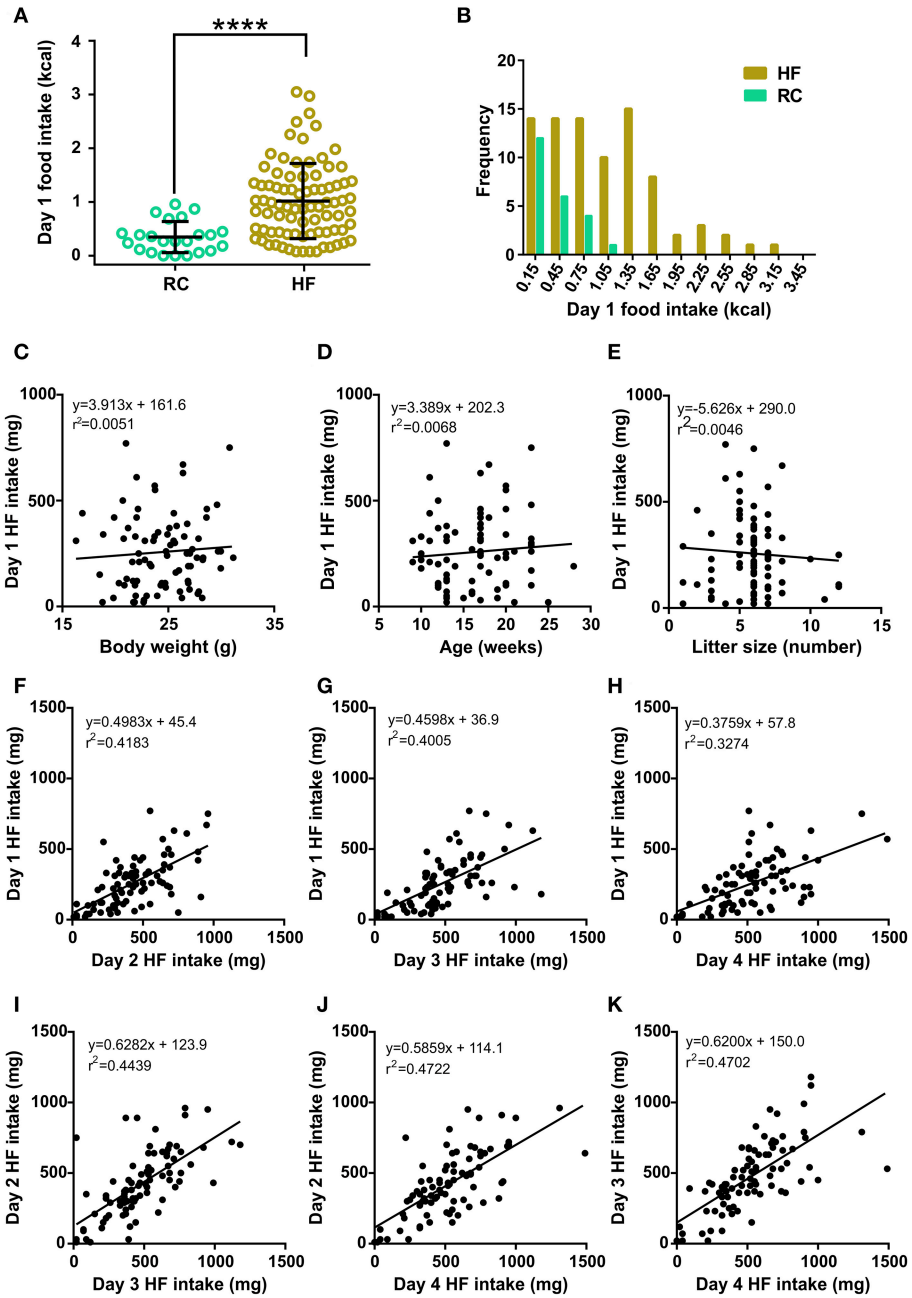

FIGURE 1 | (A) Dot plot of the individual food intake values of RC (light green circles, $n = 23$) and HF (khaki circles, $n = 84$) groups. Lines represent the mean and SD of each group. ****$p < 0.0001$, Mann-Whitney test ($U = 346.5$). **(B)** Corresponding histograms of food intake for HF and RC mice. **(C–E)** Scatter plots of day 1 HF intake vs. body weight **(C)**, age **(D)**, and litter size **(E)**. In each case, the linear regression between both parameters is shown, along with the corresponding equation and R squared. **(F–K)** Scatter plots of day 1 HF intake vs. day 2 HF intake **(F)**, day 3 HF intake **(G)**, and day 4 HF intake **(H)**; day 2 HF intake vs. day 3 HF intake **(I)** and day 4 HF intake **(J),** and day 3 HF intake vs. day 4 HF intake **(K)**. In each case, the linear regression between both parameters is shown with the corresponding equation and R squared. All linear regressions presented a slope significantly different from zero according to an F test [in **(F)**: $p < 0.0001$, $F = 58.96$; in **(G)**: $p < 0.0001$, $F = 54.78$; in **(H)**: $p < 0.0001$, $F = 39.91$; in **(I)**: $p < 0.0001$, $F = 65.43$; in **(J)**: $p < 0.0001$, $F = 73.37$ and in **(K)**: $p < 0.0001$, $F = 72.77$].

Quantitative Neuroanatomical Analysis

Blind quantitative analysis of double c-Fos+/TH+ cells was manually performed by two independent observers. Quantitative analysis was performed in sections between bregma −3.28 and −3.92 mm for the VTA. Data were expressed as the total number of c-Fos+/TH+ cells in the VTA and obtained by multiplying by four the total number of counted cells. Data were corrected for double counting, according to the

method of Abercrombie (Abercrombie, 1946) where the ratio of the actual number of neurons or cell nuclei to the observed number is represented by T/(T + h) where T = section thickness, and h = the mean diameter of the neuron or cell nuclei along the z-axis. The mean diameter of the neurons was determined using Fiji. Bright-field images were acquired with 10X/0.30 and 60X/0.80 objectives using a Nikon Eclipse 50i and a DS-Ri1 Nikon digital camera with a 0.45X adapter.

Data Processing and Statistical Analyses

Analyses were performed with a retrospective dataset coming from a combined population of 107 mice, which were tested in three independent studies following identical procedures. Part of the data has been already published (7), while the rest remained unpublished. HF group included 84 mice (36, 29, and 19 mice from studies 1, 2, and 3, respectively) and RC group included 23 mice (7, 8, and 8 mice from studies 1, 2, and 3, respectively). For each mouse, compiled information included previously recorded data (e.g., sire, mother, date of birth, litter size) as well as data of the experimental days (e.g., overnight RC intake, HF intake on successive days, body weight, date of experiment). Since no significant differences were found among studies 1, 2, and 3 for day 1 HF intake ($p = 0.0966$, Kruskal-Wallis test) or for any other analyzed parameter, all analyses were performed with data of the combined population of mice. An initial exploratory phase included the computation of a correlation matrix between variables in order to identify combinations of variables that could account for the variability of day 1 HF intake. Since day 1 HF intake positively correlated with HF intake on days 2, 3, and 4, this set of three variables was subjected to a clustering algorithm to generate a net 2-group partitioning of day 1 HF intake distribution. The classification method used was an agglomerative hierarchical clustering algorithm using a Euclidean metric for complete linkage distance. Clustering algorithms as well as the further analysis of the resulting partition were performed in KNIME (9). Statistical analysis was performed using Graphpad Prism (GraphPad Software) and differences were considered significant when $p < 0.05$. The specific statistical test for each analysis is described in the Results section. Data are presented as mean ± SEM, unless otherwise stated.

RESULTS

As we reported previously (7), day 1 food intake was significantly higher in the HF group as compared to the RC group (**Figure 1A**). Notably, food intake displayed a normal distribution in the RC group (K2 = 2.401, $p = 0.3011$, D'Agostino & Pearson omnibus normality test). In contrast, day 1 HF intake displayed a large degree of variability, ranging from 20 to 750 mg, in the HF group, and it did not adjust to a normal distribution (K2 = 9.286, $p = 0.0096$, D'Agostino & Pearson omnibus normality test, **Figure 1B**). Thus, we hypothesized that some intrinsic heterogeneity of the mice population was responsible for the increased variability observed in the spontaneous HF intake. In order to identify parameters that could be associated to the differential spontaneous HF intake, we looked for correlations between all recorded data for each mouse. No significant correlations were found between day 1 HF intake and body weight (**Figure 1C**, $F = 0.4221$, $p = 0.5177$, F test), age (**Figure 1D**, $F = 0.5586$, $p = 0.4569$, F test), litter size (**Figure 1E**, $F = 0.3782$, $p = 0.5403$, F test), time of the year, nor any other analyzed parameter in the dataset (data not shown). In contrast, we found positive and significant correlations among HF intake over the different events (**Figures 1F–K**, see F and p-values in the figure legend). These correlations suggested that the HF intake for each mouse over the consecutive days could be informative to unmask inter-individual differences in their spontaneous tendency to eat such diet.

Since day 1 HF intake did not adjust to a normal distribution, we hypothesized that the HF group contained at least two subgroups of mice with different tendencies to eat HF diet. As a maneuver to generate a net 2-group partitioning of day 1 HF intake distribution, we applied a bottom-up (i.e., agglomerative) hierarchical clustering algorithm to the HF intake of days 2, 3, and 4. Note that day 1 HF intake was explicitly excluded from this analysis. Specifically, each mouse was represented as a vector containing the corresponding values of HF intake in days 2, 3, and 4. The algorithm starts with each mouse as a single cluster and tries to combine the most similar clusters into bigger clusters until all mice are combined into two distinct groups. The similarity between clusters was determined using the complete linkage method, which defines the distance between two clusters as the maximal distance between any two members within each cluster, with the Euclidean metric as a distance measure. The application of the clustering algorithm on the HF intake in days 2, 3, and 4 allowed for a net 2-group partitioning of the HF group, in which mice can be classified into "low" or "high" HF intake groups (**Figures 2A,B**). Notably, day 1 HF intake of "low" and "high" HF intake groups appeared normally distributed (K2 = 3.606, $p = 0.1648$ and K2 = 4.881, $p = 0.0871$, respectively, D'Agostino & Pearson omnibus normality test, **Figure 2C**). "Low" HF intake group represented 34.5% of total HF diet-exposed mice and was similar to the RC group in terms of both day 1 food intake (0.46 ± 0.07 vs. 0.35 ± 0.06 kcal, $p = 0.216$, t-test) and the cumulative distributions of day 1 food intake (**Figure 2D**). "High" HF intake group showed a significantly higher day 1 HF intake (1.29 ± 0.09 kcal) as compared to both "low" HF intake and RC groups ($p < 0.0001$, Kruskal-Wallis test; $p < 0.0001$, Dunn's multiple comparisons test). Notably, both "low" and "high" HF intake groups escalated HF consumption over the successive days; however, "high" HF intake group displayed a significantly larger HF consumption than the "low" HF intake group over the successive days (**Figure 2E**). Importantly, "low" and "high" HF intake groups contained mice from studies 1, 2, and 3, and day 1 HF intake for each group did not differ among studies [Two-way ANOVA, group vs. study: interaction $F_{(2,78)} = 0.3175$, $p = 0.7289$; study effect: $F_{(2,78)} = 0.1776$, $p = 0.8376$; group effect: $F_{(1,78)} = 28.84$, $p < 0.0001$. Between-study Post-test, p-value adjusted for multiple comparisons: $p = 0.9907$, 0.9980, and

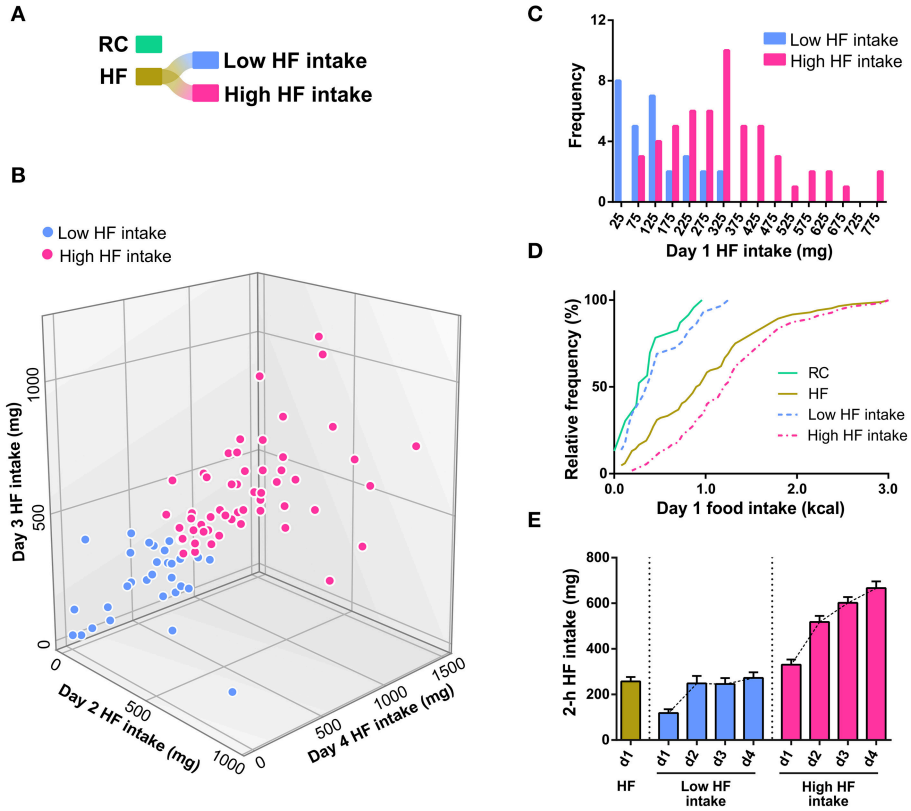

FIGURE 2 | (A) Graphical representation of the original dataset (RC and HF), and the new partitioning of HF after clustering. The coloring scheme is maintained throughout the figures. **(B)** 3D scatter plot of day 2, day 3, and day 4 HF intake classified into the two most dissimilar sets (labeled Low HF intake and High HF intake, and colored light blue and hot pink, respectively) after applying a clustering algorithm on these same days. The classification method used was an agglomerative hierarchical clustering algorithm implemented in KNIME, using the main two clusters obtained with a Euclidean metric for complete linkage distance. Note that day 1 HF intake was explicitly excluded from this analysis. **(C)** Histograms of the day 1 HF intake from the data shown in **Figure 1 (B)**, which has been disaggregated into two clusters of maximal dissimilarity (low HF intake and high HF intake) based on the HFD intake of the following 3 days. **(D)** Cumulative distributions of day 1 food intake of the RC, HF, Low HF intake and High HF intake groups. **(E)** Bar graph showing the day 1 HF intake in the HF group and HF intake observed over successive days in the Low HF intake and High HF intake groups.

0.9997 for "low" HF intake group, $p = 0.5066$, 0.9277, and 0.7621 for "high" HF intake group].

In order to gain some insights about the neurobiological basis behind the low or high tendency of C57BL/6 mice to eat HF diet, we quantified the number of VTA dopamine neurons positive for c-Fos after food intake in the last experimental day. As previously shown (4), we confirmed that the number of c-Fos+/TH+ cells of the VTA was significantly higher in HF group as compared to the RC group (**Figures 3A,B**). In the HF group, the number of c-Fos+/TH+ of the VTA positively correlated with day 4 HF intake ($F = 8.166$, $p = 0.0170$, F test, **Figure 3C**). Interestingly, a significant difference was detected when the number of c-Fos+/TH+ cells of the VTA was compared between mice clustered as "low" or "high" HF intake groups (**Figure 3D**).

DISCUSSION

Individuality refers to the collection of divergent behavioral and physiological traits among individuals of a genetically identical population (11). Inbred C57BL/6 mice are known to display inter-individual phenotypic variability for some traits. For instance, C57BL/6 mice can be classified into "low" or "high" alcohol drinkers based on the bimodal pattern of distribution of alcohol preference (12–14). Inter-individual variability in alcohol preference lacks correlation with factors such as gender, locomotor activity, age and body weight, among others (12–16). "Low" or "high" alcohol drinkers display neurobiological differences in some mesolimbic brain areas such as changes in the expression of genes related to neurotransmission or to the control of epigenetic mechanisms (14) and differential activity of VTA dopamine neurons, suggesting that they differ in their response to the rewarding properties of alcohol (16). High and low alcohol drinkers also differ in the expression of orexin receptor genes in some mesolimbic centers (17). Inbred C57BL/6 mice also show inter-individual variability in response to stress (18). For instance, male mice subjected to chronic social defeat stress can be classified into "susceptible" or "unsusceptible," based on their social interaction scores (19). Similarly, male mice can be classified into having "high" or "low" basal levels of anxiety based on their latency to emerge

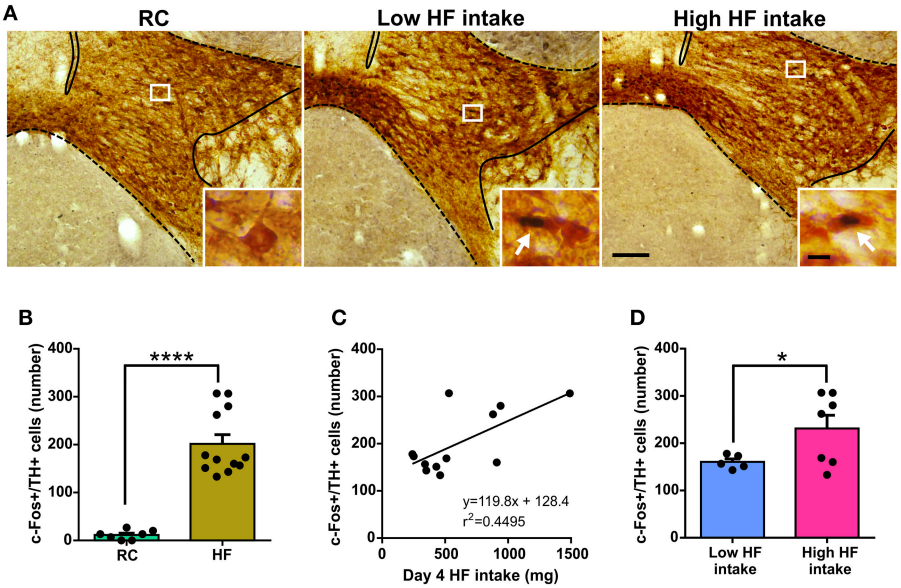

FIGURE 3 | (A) Representative photomicrographs of the double c-Fos/TH immunostaining in the VTA of RC, Low HF intake and High HF intake groups. In each picture, the limits of the VTA are overlaid according to the delineation described in the Mouse Brain Atlas (10). Insets show, in high magnification, the areas delimited by rectangles in low magnification images. Arrows point at dual-labeled cells. Scale bars: 100 μm, low magnification and 10 μm, high magnification. **(B)** Quantitative analysis of the number of c-Fos+/TH+ cells in the VTA of RC and HF groups. Bars represent the mean ± SEM and dots represent the individual c-Fos+/TH+ values for each mouse. ****$p < 0.0001$, unpaired *t*-test with Welch's correction ($t = 9.716$, $df = 11.83$). **(C)** Scatter plot of c-Fos+/TH+ cells number vs. day 4 HF intake. The linear regression between both parameters is shown with the corresponding equation and R squared. The linear regression presented a slope significantly different from zero according to an *F* test ($p = 0.0170$, $F = 8.166$). **(D)** Quantitative analysis of the number of c-Fos+/TH+ cells in the VTA of low HF intake and high HF intake groups. Bars represent the mean ± SEM and dots represent the individual c-Fos+/TH+ values for each mouse. *$p < 0.05$, unpaired *t*-test with Welch's correction ($t = 2.446$, $df = 6.639$).

from their home cage into a non-familiar environment (20). Inbred C57BL/6 mice also show inter-individual variability in their susceptibility to develop obesity after long-term HF feeding, and, consequently, they can be classified into "high" or "low" gainers (21, 22). Low gainers are resistant to diet-induced obesity and remain sensitive to the anorectic actions of leptin (23). The inter-individual variability in diet-induced obesity is mainly due to changes in fat mass. A recent study also identified that male C57Bl/6JOla mice can be classified into "intolerant" or "tolerant" depending on the length of the transient increase of food intake that they display when switched from RC to HF diet (24). Both groups of mice show similar basal RC intake or initial body weight but "intolerant" mice gain more body weight than "tolerant" mice after several weeks on HF diet (24). Interestingly, long-term HF intake seems to accentuate preexisting traits of inbred mice since the HF diet-induced body weight gain was associated to the inter-individual variability in either fat mass, body weight or expression levels of genes linked to adipose tissue expansion before exposure to HF diet (21, 23, 25). Thus, current observation that inbred C57BL/6 mice display either a low or a high tendency to eat HF diet represents another trait in which this mouse strain shows inter-individual phenotypic variability.

HF intake in our experimental conditions is presumably driven by the palatable nature of the stimulus since it occurs in satiated mice, at a time of the day when spontaneous food intake is minimal, and while mice remain with free access to RC (1). It seems likely that HF intake is linked to fat-related organoleptic properties of the stimulus (e.g., flavor) because the fat content in the employed HF diet is increased at the expense of a reduction of the other macronutrients. Interestingly, the tested HF diet represents a mild rewarding appetitive stimulus as the fat content is moderately high. as compared to some commercial HF diets (26), and it does not contain added sucrose. Such mild nature of the tested stimulus may have helped to unmask the presence of the inter-individual variability in HF intake as it could be predicted that a highly palatable diet would have promoted strong food intake in the vast majority of mice.

As previously shown (4), we confirmed that daily and time-restricted HF intake in mice activates VTA dopamine neurons. Here, we also found that the number of activated VTA dopamine neurons of mice daily exposed to HF diet positively correlated with day 4 HF intake, and that mice clustered as "high" HF intake group displayed a higher number of activated VTA dopamine neurons as compared to numbers found in mice clustered as "low" HF intake group. Since HF intake *per se* activates VTA dopamine neurons and, in turn, activation of VTA dopamine neurons increases HF intake, the cause-effect relationship between these two findings remains uncertain. In a previous study, we found that the number of activated dopamine neurons of the parabrachial pigmented and interfascicular

sub-regions of the VTA was higher after day 4 HF intake as compared to day 1 HF intake suggesting that these neurons were responsive to the amount of HF diet ingested (4). However, we also found that dopamine neurons of the interfascicular sub-region of the VTA were activated in anticipation to HF intake on day 4 suggesting that some VTA dopamine neurons may be also linked to the mechanisms driving HF intake in mice that are trained to daily receive it (4). Thus, our results indicate that some VTA dopamine neurons are involved in the HF intake under these experimental conditions, but their potential role as neurobiological substrates of the inter-individual variability requires future studies. In addition, further studies are required to evaluate the potential involvement of other mesolimbic areas as potential candidates mediating a different tendency of C57BL/6 mice to eat HF diet.

Since inbred mice are presumably isogenic, the presence of inter-individual variability of some traits is therefore attributable to factors that each mouse experiences, such as stochastic events or unique environmental circumstances (Champagne, 2013). Environmental factors impacting on the inter-individual variability are countless and could act intra-uterus (e.g., uterine position and blood supply, size of the litter, sex of the neighboring fetus), in the early postnatal period (e.g., size and sex ratio of the litter, maternal behavior, social interactions, stress) and/or in adulthood (dominance status, stress, health issues). A detailed description of these factors and its impact on inter-individual variability is well-described in specific reviews (27–31). Some of these environmental factors favor inter-individual variability via induction of epigenetic modifications of the genome (e.g., DNA methylation, histone acetylation, or microRNA modifications) that end up altering gene expression (11, 32). Notably, the tendency to consume HF diet was shown to be affected by environmental factors via epigenetic modifications. For instance, offspring from inbred C57BL/6 dams that consume HF diet during pregnancy and lactation show increased sucrose and fat preference in adulthood (33). Changes in the tendency to consume palatable foods were linked to a reduction in DNA methylation of some gene promoters of the dopamine and opioid systems that alters its long-term gene expression in the mesolimbic pathway (33).

The factor/s that determine the individual variability in the spontaneous tendency to consume HF diet in our experimental conditions is currently uncertain. Our analysis argues against some key factors, such as litter size, body weight or age, playing a major contribution to the individual tendency to display low or high HF intake. It is important to mention that the current study does not allow us inferring if such inter-individual phenotypic variability is due to intrinsic differences in feeding behavior or secondary to an inter-individual phenotypic variability of other mouse traits. For instance, differences in HF intake may be secondary to different anxiety/stress responses of mice to the experimental paradigm or secondary to variations in their natural biological rhythm (i.e., sleep/wakefulness, arousal, circadian rhythms) that affect food intake in the light period. Further studies are necessary to evaluate a causal association among one or more environmental factors and the tendency to eat specific types of foods.

It is important to mention that unmasking inter-individual phenotypic variability usually requires a high number of animals, in order to be more confident that a non-normal distribution of the population is not a consequence of a small sample size. Here, we analyzed combined data from 84 mice exposed to HF diet. The above-referred studies by Yang et al. (22), Koza et al. (21), or Enriori et al. (23) describing phenotypic variability for other traits in inbred C57BL/6 mice used 277, 107, or 178 mice, respectively. Additionally, it is interesting to point out that different criteria have been used to classify animals into different subgroups in a given population. For instance, Krishnan et al. (19) set a cutoff to divide "susceptible" and "unsusceptible" mice based on the social interaction scores of control mice, while Brenachot et al. (24) used a median-based split on 1-week cumulative HF intake to separate "intolerant" and "tolerant" mice. Since mice displaying a potentially different tendency to consume HF diet showed considerable overlap in day 1 HF intake, we chose to apply a hierarchical clustering algorithm on the HF intake in successive days in order to have a 2-group partitioning of day 1 HF intake. Such strategy allowed us to unmask the presence of a group of mice that displayed a HF intake on day 1 similar as seen in the control group, and that consistently showed a lower tendency to consume HF diet over the successive experimental days as well as a smaller activation of VTA dopamine neurons.

Despite the non-genetic neurobiological basis behind the inter-inter-individual variability for HF intake remains uncertain, it is intriguing to speculate the implications of the observations herein discussed on human physiology. Twin and adoption studies have clearly shown significant genetic influences on body mass index, food intake and intake patterns; however, evidence of heritability for food preferences is less strong (34). Notably, a study found that obese twins showed higher preference for fatty foods than the lean co-twin suggesting that the acquired preference for fatty foods is associated with obesity, independently of genetic background (35). Since intake of palatable foods (e.g., sugar-sweetened drinks) positively correlates with body mass index, fat mass and waist circumference (36), it seems clear that the implementation of a comprehensive nutrition education that improves eating habits toward the preference/acceptance of healthy foods would help against the worldwide obesity epidemic.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the article reports the analysis of internal data of the laboratory. We can make the data publicly available if needed. Requests to access the datasets should be directed to mperello@imbice.gov.ar

AUTHOR CONTRIBUTIONS

MC, PD, and MP contributed to the conception and design of the study. MC, PD, FB, GG, and MA organized the database. PD and MC performed the statistical analyses. SV, MC, FB, and

GG obtained the experimental data. MC and MP wrote the first draft of the manuscript. MC, PD, FB, GG, MA, and MP wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Activity Based Anorexia as an Animal Model for Anorexia Nervosa–A Systematic Review

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Anorexia nervosa (AN) is a severe eating disorder affecting around 1 per 100 persons. However, the knowledge about its underlying pathophysiology is limited. To address the need for a better understanding of AN, an animal model was established early on in the late 1960's: the activity-based anorexia (ABA) model in which rats have access to a running wheel combined with restricted food access leading to self-starving/body weight loss and hyperactivity. Both symptoms, separately or combined, can also be found in patients with AN. The aim of this systematic review was to compile the current knowledge about this animal model as well as to address gaps in knowledge. Using the data bases of PubMed, Embase and Web of science 102 publications were identified meeting the search criteria. Here, we show that the ABA model mimics core features of human AN and has been characterized with regards to brain alterations, hormonal changes as well as adaptations of the immune system. Moreover, pharmacological interventions in ABA animals and new developments, such as a chronic adaptation of the ABA model, will be highlighted. The chronic model might be well suited to display AN characteristics but should be further characterized. Lastly, limitations of the model will be discussed.

Keywords: eating disorder, food restriction, hypophagia, hyperactivity, mice, rats, self-starvation, weight loss

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INTRODUCTION

Anorexia nervosa (AN) is characterized by underweight, self-induced weight loss achieved by food restriction or increased physical activity, endocrine alterations, and disturbance of body image affecting mostly young women with a 12-month prevalence of 0.4% among adolescents and young adults (1). The loss of body weight resulting in body mass index (BMI) values under 17.5 kg/m^2 leads to various somatic symptoms affecting humoral and central nervous signaling as well as cardiovascular and gastrointestinal functions (2). To fulfill the diagnostic DSM-V criteria of anorexia nervosa a patient must show a restriction of energy intake inducing low body weight, a fear of gaining weight or behavior preventing weight gain as well as a disturbance of body image or lack of understanding of the danger of low body weight. Two subtypes of anorexia nervosa can be distinguished: restrictive AN characterized by restrictive calorie intake and binge-purging AN with self-induced vomiting (or other means to purge). It is to note that patients with AN often have psychiatric comorbidities such as affective, anxiety, obsessive-compulsive, and substance abuse disorders (3). These complications account for the high annual mortality rate of the disease, namely of 5.4 deaths per 1,000 person-years, representing the highest mortality rate among all psychiatric diseases (4).

Therapeutic interventions encompass nutritional and psychotherapy as well as—where applicable—drug therapy; however, the relapse rate is high ranging between 35 and 41% within 18 months (5). Consequently, there is a need for more efficient therapeutic options which requires better understanding of the underlying pathomechanisms responsible for the development of AN. To increase the knowledge about diseases, animal models depicting alterations of the disease can be very helpful.

In the late 1960's, core features of AN, namely hyperactivity, reduction of food intake and weight loss were observed in rats exposed to food restriction combined with access to a running wheel. Those animals increased their activity (6) and reduced food intake (7) leading to self-starvation and hyperactivity further aggravating weight loss. This negative relationship between food intake and activity seemed counterintuitive at first; however, subsequently it was hypothesized that an increase of activity allows animals/individuals to reach an area with stable food sources, thereby securing survival (8). Moreover, hyperactivity was also shown to suppress appetite (8) and to be an attempt to avoid a drop of body temperature (9). Additionally, in patients suffering from AN the drive for hyperactivity is positively correlated with anxiety, suggesting increasing activity as a mean to reduce anxiety (10).

Finally, the observation of self-starving in male rats which had unlimited access to a running wheel and restricted (1 h/day) access to food led to the establishment of ABA as a model of AN (11). Noteworthy, food-restricted male animals without a running wheel stabilized their body weight on a lower level, pointing toward the great importance of activity in this animal model (11). Consequently, the model was termed activity-based anorexia (ABA), sometimes also called exercise-induced anorexia or food restriction-induced hyperactivity (11). The standard ABA protocol consists of a 1–2 h feeding period during the light or dark phase, combined with a 22–24 h wheel access (11), leading to increasing hyperactivity and decreasing food intake over time. In contrast, when food access is not restricted in time but a fixed amount of food is given in the "semi-starvation-induced hyperactivity" model, no (further) reduction in food intake can be observed as indicated by similar intake of food in food restricted animals with and without access to a wheel (12). Thus, only ABA depicts a reduction of food intake/loss of appetite as observed in human AN. Most of the more recent ABA protocols include an acclimatization period to the wheel prior to the start of food restriction. The ABA protocol is usually pursued until a certain weight loss criterion, frequently defined between 15 and 30% weight loss (11), is reached. Additionally, a well-established variation of the ABA protocol is the gradual reduction of food access over several days (13). Most recently, several protocols describe prolonged endurance of food restriction and wheel exposure over a period of 55 days (14).

The present systematic review will present the current knowledge on the ABA model, discuss alterations induced by the model, highlight pharmacological options tested under these conditions and also mention limitations of the model. Lastly, gaps in knowledge will be addressed to stimulate further research.

METHODS

For the systematic data search the PRISMA guidelines were applied. The data bases Pubmed—Medline and Web of Science were searched using the following search terms: "Activity based anorexia," "Semi starvation induced hyperactivity," "Exercise induced anorexia," and "Food restriction induced hyperactivity." Additionally, the EMBASE database was searched using the terms "Anorexia nervosa AND rat" and "Anorexia nervosa AND mouse." The search was performed on October 20th 2018. Selection criteria applied were original publications, animal studies and English language. Thus, the exclusion criteria encompassed reviews, editorials, human studies, und manuscripts written in another language than English. In the manual screening all publications were identified investigating rodents exposed to food restriction in combination with running wheel access. Studies lacking one of these two characteristics were not included into the final list of publications. However, some are still mentioned in the manuscript to provide background information or are used for comparison. Several additional references were identified through review of the publication lists of the included articles for background information. Of those, nine were included into the final selection of references. After selection, 102 publications were included in this systematic review (**Figure 1**).

INFLUENCING FACTORS

Various versions of ABA protocols were used so far as presented in **Table 1**. Consequently, several influencing factors such as pre-exposure, ambient temperature and sound, handling and maternal separation, diet and food access, activity, sex, strain and genetics have been described able to affect food intake, activity and thus body weight loss during the development of ABA. These influencing factors along with the outcomes are summarized in detail in **Table 2**.

The following main conclusions can be drawn: Maternal separation and daily handling should be omitted or be similar for all animals, since it significantly influences weight loss (51, 52). ABA occurs because wheel running interferes with adaptation to the feeding schedule, which is omitted when the feeding

Abbreviations: 2DG, 2-deoxy-D-glucose; 5-HT, serotonin; α-MSH, alpha-melanocyte stimulating hormone; ABA, activtiy-based anorexia; ACTH, adrenocorticotropic hormone; AgRP, Agouti-related protein; AN, anorexia nervosa; ARC, arcuate nucleus; BDNF, Brain-derived neurotrophic factor; BMI, body mass index; CART, cocaine- and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; DMH, dorsomedial hypothalamus; ER, estrogen receptor; GABA, Gamma-aminobutyric acid, GHS-R1a, growth hormone secretagogue receptor 1a; HPA axis, hypothalamus-pituitary-adrenal axis; icv, intracerebroventricular; IGF-2, insulin-like growth factor 2; ip, intraperitoneal; LC, locus coeruleus; LEPR, leptin receptor; LHA, lateral hypothalamus; NAc, nucleus accumbens; NMDA, N-methyl-D-aspartic acid; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; POMC, Pro-opiomelanocortin; PVN, paraventricular nucleus; sc, subcutaneous; SD, Sprague Dawley; SSRI, selective serotonin reuptake inhibitor; TLR4, Toll-like receptor 4; VTA, ventral tegmental area.

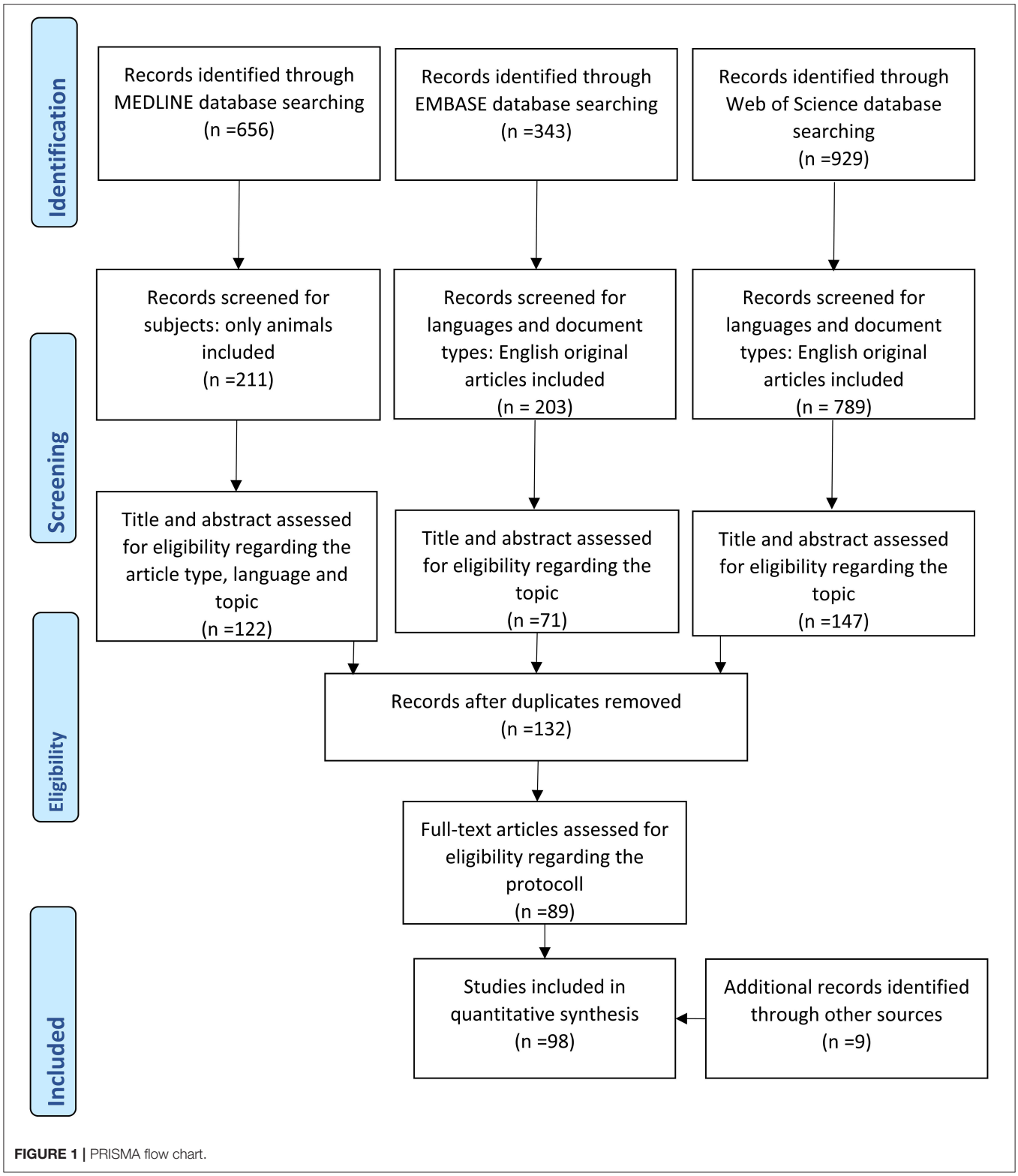

FIGURE 1 | PRISMA flow chart.

schedule is introduced before the running wheel. Warm ambient temperature could have an ameliorating effect on AN; thus, a standard ambient temperature should be used in ABA to reach the weight loss criterion. A feeding schedule for <2 h in rats with standard chow and *ad libitum* access to water is sufficient to induce hyperactivity and weight loss, both indicators

TABLE 1A | ABA protocols and respective outcomes (≥3 similar protocols of one research group).

Species	Food	Wheel access	Acclimatization	Duration	Body weight loss	References
Male C57Bl/6J mice	Progressive limited food	Free access	5 days of wheel	12 days	25%	(13)
	Access from 6 h/day (day 1) to		Acclimatization		23%	(15)
	3 h/day (day 4) at beginning of				26.7%	(16)
	Dark phase				22.5%	(17)
					22.7%	(18)
Female C57BL/6J mice					12%	(15)
					15%	(19)
				5 days and 12 days	12 and 15%	(20)
				5 days and 9 days and 12 days	12 and 16% and 14%	(21)
Female adolescent Sprague Dawley (SD) rats	1 h/day at the onset of dark phase	Free access	48 h of wheel acclimatization	3 days	16.7%	(22)
			3 days of wheel acclimatization	5 days	17.9%	(23–26)
				3 days	11.5%	(27)
				2–4 days	25%	(28)
			24 h of wheel acclimatization		20%	(29)
			4 days of wheel acclimatization	5 days	13.3%	(30)
				4 days	13.3%	(31)
Female C57Bl/6J mice	1 h/day at the onset of dark phase		2–3 days wheel acclimatization	2 days	22.9%	(32)
	2 h/day at the onset of dark phase		4 days of wheel acclimatization	3 days	22.2%	(33)
Male mice			5 days of wheel acclimatization	4 days	22.3%	(34)
Male and female C57Bl/6J mice					Male: 26.3% Female: 27%	(35)
Male adolescent SD rats	1.5 h/days	22.5 h	None	4 days	25 %	(36)
	1.5 h/day at the onset of light phase			3.1–3.2 days	26%	(37)
				3 days	26%	(38, 39)
				5 days	25%	(40)
				Not mentioned	25%	(41–43)
SD rats				male: 4 and 5 d, female: 6 and 7 days	25 and 30%	(44)
Female Long–Evans rats	2 h/day at the onset of dark phase	Free access	None	6 days	25%	(45)
				5 days	25.9%	(46)
	2 h/day during dark phase		10 days of wheel acclimatization	5 days	23%	(47)
Female SD rats	1.5 h/day at the onset of dark phase	Free access	10 days of wheel acclimatization	6 days	12%	(48)
Female adolescent SD rats				4 days	17.2%	(49)

(Continued)

TABLE 1A | Continued

Species	Food	Wheel access	Acclimatization	Duration	Body weight loss	References
	2 h/day at the onset of dark phase for 5 days, then 1 h/day at the onset of dark phase 1 h/day at the onset of dark phase		7 days of wheel acclimatization 10 days of wheel acclimatization	10 days	22.4% 29% lost 25% 86% lost 25%	(50)
SD rats	1.5 h food access during light phase	22.5 h	2h/2 days of wheel acclimatization	Male: 7 days; Female: 6 days	25%	(51)
Wistar rats				Male: 3 days; Female: 7 days	25%	(52)
Female SD rats			2 h/3 days of wheel acclimatization	4–7 days	20%	(53)
Female SD rats Male SD rats			2 h/day for 3 days of wheel acclimatization	5 days 4 days	21.3% 17.9%	(54)
Male albino Wistar rats	1.5 h during light phase	22.5 h	2 h/4 days of wheel acclimatization	7 days	25%	(55)
Male SD rats			2 h/3 days of wheel acclimatization	3–7 d (median 4 days)	25%	(56)
				5–9 days	20%	(57)
Female C57Bl/6J and Female DBA/2J mice	2 h/day at the onset of dark phase	Free access	3 days of wheel acclimatization	4 days	15% 22.5%	(58)
			7 days of wheel acclimatization	3 days	17.8% 10.5%	(59)
Female KK mice Female AKR mice m Female NZW mice Female A/J mice Female C3H mice Female C57BL mice Female WSB mice Female BALB mice Female CAST mice Female FVB mice Female DBA mice Female Wistar rats	5 pellets of about 1.2 g 2 h at the beginning of the dark phase 12 pellets of about 1.2 g 1.5 h at the beginning of the dark phase	None	7 days of wheel acclimatization 10 days of wheel acclimatization	4 days 5 days	100% lost 15% 100% lost 15% 100% lost 15% 88% lost 15% 85% lost 15% 80% lost 15% 30% lost 15% 28% lost 15% 25% lost 15% 15% lost 15% 0% lost 15% 0% lost 25%	(60)
Female Wistar rats	1 h/day at the onset of dark phase	Free access	10 days of wheel acclimatization	4 days	17.5%	(61)
			15 days of wheel acclimatization	6 days	24.2%	(9)
			10 days of wheel acclimatization	7 days	22.5%	(62)
	2 h/day and 1h/day			6 days	19.2 and 29.3%	(63)
	1 h/day at the onset of dark phase			7 days	25%	(64)
				5 days	18.7%	(65)
				7 days	18.3%	(66)

(Continued)

TABLE 1A | Continued

Species	Food	Wheel access	Acclimatization	Duration	Body weight loss	References
Female Balb/cJ mice	6 h/day from during light phase	Free access	9 days of wheel acclimatization	6 days	22.5%	(67)
				4 days	25%	(68)
Female Balb/cJ mice and	2 h/day during light phase		7 days of wheel	4 days	30%	(69)
Female A/J mice	2 h		acclimatization	6 days	25%	
Female Balb/cJ mice	4 h		9 days of	3 days	27.5%	
	6 h		wheel acclimatization	3 days	27.5%	
	8 h			3 days	25%	
	10 h			11days	10%	
				11 days	5%	
Female Wistar rats	1.5 h/day during light phase	Free access	None	7 days	15%	(70)
Male Wistar rats				9 days	20%	(71)
Wistar rats	1 h/day during light phase			5 days	15%	(72)
Male and female Wistar rats	1.5 h/day at the onset of light phase	22.5 h	Not mentioned	Male: 9 days; Female: 10 days	20%	(73)
Female SD rats	1.5 h during light phase	Free access	7 days of wheel acclimatization	14 days	22%	(74)
					23.0%	(75)
					25%	(76)
Female Wistar rats	1.5 h/day at the onset of dark phase	Free access	10 days of wheel acclimatization	6 days	20%	(77)
				4 days	21%	(78)
				6 days	17.3% 14.2%	(79)
	1 h/day at the onset of dark phase 1 h/day at various time points during dark phase			4 days	13.4% 17.4%	(80)
Female Wistar rats Female C57Bl/6 mice	1 h/day at the onset of dark phase 2 h/day at the onset of dark phase		10 days of wheel acclimatization 5 days of wheel acclimatization	5 days 3 days	25% 22%	(81)

TABLE 1B | ABA protocols and respective outcomes (<3 similar protocols).

Species	Food	Wheel access	Acclimatization	Duration	Body weight loss	Study
Female SD rats	1 h/day immediately prior to the dark phase	23 h	1.5 weeks of wheel acclimatization	5–18 days	20%	(82)
Female SD rats	1.5 h/day in middle of light phase	22.5 h	1 day of acclimatization to food restriction	10 days	18.9%	(83)
Male albino rats	1 h/day	22.5 h	1 day of acclimatization to food restriction	6 days	16%	(84)
Male SD rats	1 h/day during dark phase	23 h	3 days of acclimatization to food restriction	9 days	25%	(85)
Male and female rats	1.5 h/day	22.5 h	None	Male and Female: 4 days	25%	(86)
Female JCR:LA-cp rats	1.5 h/day at the onset of dark phase	22.5 h	10 days of acclimatization to food restriction	5 days	22%	(87)
Male JCR:LA-cp rats	1.5 h/day at the onset of dark phase	22.5 h	None	10 days	27%	(88)
Male Lewis rats Male Brown-Norway rats Male Fischer 344 rats	1.5 h/day during light phase	22.5 h	None	8 days 7 days 9 days	25.2% 24.7% 26.3%	(89)
Male SD rats	Food and water for 1.5 h/day during light phase	free access	None	4–14 days	12.1%	(90)
Male Wistar rats	1 h/day at the onset of dark phase	23 h	7 days of wheel acclimatization	7 days	25%	(91)
Female ICR mice	At the onset of dark phase	0.5 h/5 days /week	None	21 days	25.6%	(92)
Female SD rats	1.5 h/day at the onset of dark phase	free access	5 days of wheel acclimatization	5 days	22.5%	(93)
Female SD rats	1.5 h/day at the onset of dark phase	free access	11 days of wheel acclimatization	4–6 days	70% lost 20%	(94)
Female SD rats	1 h/day during dark phase	23 h	-	4–7 days	74%	(95)
Female Long-Evans rats	1 h/day during dark phase	Free access	7 days of wheel acclimatization 25 days of wheel acclimatization	3–6 days 5 days	20% 15%	(96)
Fem. adolescent Long-Evans rats	1 h/day before onset of dark phase	Free access	8 days of wheel acclimatization	4 days	40%	(97)
Male SD rats	90 min during light phase	22.5 h	7 days of acclimatization to food restriction	7 days	15.3%	(98)
Male C57/BL6J mice	3 h/day restriction proceeded as follows: 6 h/day on day 1.5 h/day on day 2, 4 h/day on day 3 and 3 h/day on day 4 progressive restrictive feeding schedule (6 h/day) decreasing by increments over a protracted period (21 days) to 2 h day	Free access	9 days of wheel acclimatization 7 days of wheel acclimatization 17 days of wheel acclimatization	7 days 18 days 40 days	30% 28%	(99)
Male SD rats	1 h/day	23 h	None	6 days	28%	(100)
SD rats	1 h/day 4 h within the light phase	Free access	7 days of wheel acclimatization	6 days	Male: 30%; Female: 20%	(101)
Female Wistar rats	1 h/day during light phase fixed time variable time point	23 h	2 days of wheel acclimatization	9 days	21% 28%	(102)
Male SD rats	1 h/day at the onset of dark phase	23 h	Pre-exposed to food restriction Pre-exposed to wheel non-exposed	2 days 8 days 10 days	1% 31.5% 27.3%	(103)

(Continued)

TABLE 1B | Continued

Species	Food	Wheel access	Acclimatization	Duration	Body weight loss	Study
Female SD rats	1.5 h/day at the onset of dark phase	Free access	7 days of wheel acclimatization	6 days	21%	(104)
Male and female ICR/CD1 mice	Gradual food restriction in the form of 3–4 h/day during dark phase	Free access	7 days of wheel acclimatization	5 days	23% 60% resistant 40% ABA	(105)
Female SD rats	2 h/day at the end of light phase (30g)	Free access	10 days of wheel acclimatization	10 days	23%	(106)
Female Wistar rats	2 h/day during dark phase 3 h/day during dark phase 4 h/day during dark phase	Free access	3 days of wheel acclimatization	21 days	18.5% (day 8) 5/10 died 11.1% weight gain (day 21) No weight loss No weight loss	(107)
Male Wistar rats	Increasing amounts of food (6–10 g) to keep weight loss between 5 g and 8 g/day during dark phase	Free access	None	10 days	31%	(108)
Male SD rats	3 h feeding period	Free access	7 of day of wheel acclimatization	7 days	12.5%	(109)

of ABA. It can be assumed that it is useful to study activity levels during the wheel adaption period in order to early on detect low susceptibility to ABA (60, 101). Obviously, wheel access is a crucial characteristic of ABA. Since pre-prandial activity increases weight loss (119), food-anticipatory activity seems to be an important factor for weight loss as well. Although in humans AN is more prevalent in females, early studies often used adult male rats to avoid the influence of hormones associated with both development and reproduction. However, in rats studies focusing on the effect of sex on ABA outcome showed that ABA seems to develop more effectively and rapidly in males; noteworthy, also food-anticipatory activity was more pronounced in males (13, 15). Since females take longer to reach the weight loss criterion (44), in order to mimic and better understand human AN, female animals should be examined in a more long term protocol. Comparing different ages, younger rats develop ABA more rapidly (123). To develop ABA in an adult rodent population takes substantially longer than in adolescence, probably resulting from the fact that—besides absence of significant body weight gain during this period—escalation of running is often blunted in adults compared to adolescent rats. As a consequence, adult animals can often be maintained in the paradigm for up to several weeks without reaching the maximum weight loss criterion. However, since younger animals are more vulnerable to ABA, young animals typically cannot be maintained in the paradigm for more than several days regardless of sex. Regarding the investigation of different strains, C57BL/6J mice might not be the first choice to examine effects of ABA (58), and A/J mice might be more useful in long-term protocols (69). Similar strain comparisons should be performed in rats.

EFFECTS OF ABA

Alterations of Hypothalamic Nuclei and Transmitters

One of the main roles of the hypothalamus is to regulate hunger, satiety, energy metabolism and ultimately body weight (124). Areas of the hypothalamus involved in food and water intake regulation encompass the arcuate nucleus [ARC, (125)], the dorsomedial hypothalamus (126), lateral hypothalamus [LHA (128)] the paraventricular nucleus [PVN (129)] and ventral medial hypothalamus (130); thus, the effects of ABA on the activity of the hypothalamus and its transmitters were studied extensively.

ABA in female rats activated neurons in the supraoptic nucleus [expressing oxytocin, related—among others—to anxiety (131)] and ARC [participates in the regulation of food intake as mentioned above (125)] compared to *ad libitum* fed rats as assessed using the neuronal marker c-Fos when perfused directly after the feeding period (74). Food-anticipatory activity in the running wheel correlated with c-Fos expression in the dorsomedial hypothalamus [DMH, involved in the regulation of food and water intake as well as body weight (126)] in female ABA Wistar rats (80). Interestingly, ABA rats on a random feeding schedule, which did not develop food-anticipatory behavior, displayed a negative correlation between neuronal

TABLE 2 | Factors influencing ABA outcome.

Influencing factor	Effects on ABA outcome
Pre-exposure to:	↑ Survival rate of 75% (110)
Restricted feeding	Diminished food intake reduction, hyperactivity and body weight loss (103)
Feeding schedule	↓ Body weight loss (111)
	↓ Food intake reduction, hyperactivity and body weight loss (103, 111)
Food restriction-induced weight reduction	No effect on ABA development (98)
Low initial body weight	No effect on ABA development (112)
Running wheel	↑ ABA vulnerability (113)
	Deaccelerated self-starvation, no effect on percentage of survival (110)
	↑ Body weight loss and hyperactivity, food intake reduction (111)
	↑ Body weight loss and hyperactivity, food intake reduction (103)
High ambient temperature	Deaccelerated body weight loss in male rats (55)
Access to a warm plate	↓ Hyperactivity and body weight loss in male rats (56)
	↓ Hyperactivity and body weight loss, food intake reduction (57)
	Reversed hyperactivity, preserved food intake in female rats (53)
	↓ Hyperactivity and body weight loss in female rats (9)
Sound attenuation condition	Extended ABA duration, ↓ hyperactivity (54)
Daily handling	↓ Body weight loss (52)
Maternal separation of 180 min daily for 20 days postnatally	Delayed reaching the removal criterion of 20% weight loss (52)
	↑ ABA resistance (greater survival) (51)
Maternal separation for 180 min/day for 14 days	Accelerated weight loss, ↑ activity, ↓ food intake in females (114).
	Prevented ABA in males (115).
Food presentation at irregular times	↑ body weight loss and hyperactivity, food intake reduction (102)
Different food access durations	6 h/day: ↓ survival (69)
Time of food presentation	3 or 4 h/day: cessation of estrous cycle, body weight loss, food intake suppression and hyperactivity (107)
Food type	2 h/day: severe gastric lesions, ↑ mortality (107).
Drinking	At the onset of dark phase: prevented body weight loss (111).
	High fat chow/vegetable fat: prevented reaching of removal criterion (85)
	0.88 M sucrose: prevented hyperactivity and body weight loss (116)
	Palatable food (2 h/day): binge eating (22).
	Wet mash/adaptation to drinking schedule: prevented ABA (117)
Running wheel access	Wheel inaccessibility 4 h before feeding: diminished body weight loss (111)
Pre-prandial/ food-anticipatory activity	Induced sickness (118)
Postprandial activity	Activity levels before ABA induction strongly predicted outcome of ABA (60, 101)
	↓ Body weight loss (119)
	↑ Body weight loss (119)
Female sex	↑ Food intake, hyperactivity, deaccelerated body weight loss (44)
Male sex	↑ Body weight loss and hyperactivity, no ↑ vulnerability to ABA (120)
	↑ Body weight loss and hyperactivity (121)
	↓ Body weight loss and food intake reduction, ↑ hyperactivity during food intake period and post-prandial hyperactivity (13, 15)
	↓ Hyperactivtiy during food intake period (35).
	↑ Food intake reduction, hyperactivity, accelerated body weight loss (44)
	Body weight loss correlated with ↑ running (120)
	↑ Mortality rate (20% weight loss in 3 days), body weight loss and food intake reduction, ↑ food-anticipatory/pre-prandial activity (13, 15)
	↓ Food-anticipatory/pre-prandial activity (35)
Strain	C57BL/6J mice: ↓ hyperactivity, DBA/2J mice: ↑ hyperactivity (58)
Genetics	Brown Norway and Lewis rats: ↓ thymus weight (89)
	A/J mice: longer survival (69).
	Chromosome substitution strains 4, 12, 13: ↑ hyperactivity during the light phase hours/food restriction phase (122)
	Lean-prone rats: ↑ hyperactivity and accelerated body weight loss (87)
	Leptin receptor deficiency: prevented reaching weight loss criterion (87)
	α4βδ-GABAAR KO female rats: ↑ body weight loss and hyperactivity, food intake reduction (35)
	Reduced miR-340 expression: ABA resistance (105)

↑, *increase;* ↓, *decrease.*

activity in the ARC and body weight loss (80). Before food access and during pre-prandial activity, c-Fos expression and ARC, PVN as well as in the nucleus accumbens [NAc, involved in the processing of motivation, aversion and reward, lesions result in increased food intake and weight gain; (127)] was observed to be reduced in male rats undergoing ABA when provided access to

0.88 M sucrose, but not after 0.002 M saccharin in a comparable sweetness, giving rise to an attenuating effect of sucrose on wheel running possibly induced by inhibition of hypothalamic activation through corticosterone (116).

Lateral hypothalamus electrical stimulation (100 Hz and 25, 50, and 75% of the maximal stimulation amplitude) on consecutive days during four test sessions significantly decreased locomotor activity in female ABA rats with no effect on food intake or survival time (72). Future studies should focus on other brain areas targeted by electrical stimulation to alter features of AN.

In female mice ABA induced an increase of hypothalamic protein synthesis. These changes of the hypothalamic proteome especially affected proteins involved in glycolysis or in citric acid cycle (21), giving rise to an adaptively enhanced energy metabolism. Additionally, proteins limiting oxidative stress were altered (21). DNM1L, implicated in mitochondrial fission, was overexpressed under conditions of ABA leading to an increased number of mitochondria, while increased dynamin-1 and LC3II/LC3I ratio indicated an activation of autophagy (21). Taken together, ABA leads to an adaptation of the hypothalamic proteome inducing autophagy and mitochondrial changes.

Pro-Opiomelanocortin (POMC), Cocaine- and Amphetamine-Regulated Transcript (CART), Agouti-Related Protein (AgRP), and Neuropeptide Y (NPY)

The protein POMC, cleaved into various active peptides such as melanocyte-stimulating hormones, is essential in the regulation of food intake inducing satiety; thus, its absence can lead to obesity (132). Among others, POMC is expressed in the hypothalamus (133). In female ABA rats, arcuate expression of POMC protein was reduced 2-fold (61). In contrast, another study reported a transient up-regulation of POMC mRNA levels in the ARC, pointing toward increased anorexigenic signaling in female ABA rats (64). This discrepancy might be due to dynamics of peptide expression in the course of ABA development. Similarly, food restriction 1 h food access per day at the beginning of the dark phase), regardless of the level of physical activity, elevated melanocortin receptor (involved in POMC signaling) expression in the ventral medial hypothalamus (134). This is likely of physiological importance as intracerebroventricular (icv) infusion of AgRP, an orexigenic peptide expressed in the ARC with inverse agonistic activity on melanocortin 3 and 4 receptors (135), increased the survival rate by decreasing physical hyperactivity and blunting suppression of food intake (134). Conversely, chronic administration of alpha-melanocyte stimulating hormone (α-MSH), an anorexigenic cleavage product of POMC (136), into the lateral ventricle stimulating the melanocortin receptors and the hypothalamus-pituitary-adrenal (HPA) axis [mediating the response to stress, regulating digestion, and energy expenditure as well as inhibiting reproductive functions (137)] elevated running wheel activity and reduced food intake in rats resulting in higher vulnerability to develop ABA (63). Arcuate protein expression levels of AgRP and NPY [orexigenic hypothalamic neuropeptide (138)] were elevated 5-fold and correlated negatively with relative body weight and white adipose tissue mass (61). A recent study corroborated these data by showing that ABA in female Sprague Dawley rats led to higher NPY, AgRP but also POMC expression (48). Additionally, increased levels of NPY mRNA in the ARC were observed in female ABA rats compared to those having *ad libitum* access to food (139). In female rats exposed to a feeding time of 2 h/day; daily icv infusion of NPY at the end of the light phase over a period of 7 days accelerated development of ABA by reducing food intake and increasing wheel running (139). Combination of food restriction and enhanced physical activity in male C57BL/6J mice elevated hypothalamic protein expression of AgRP, while food restriction alone increased NPY and AgRP, indicating that anorexic-like conditions disrupt hypothalamic circuitries (140). Therefore, it can be hypothesized that ABA induces alterations of neuropeptides including AgRP, NPY, POMC, and CART in first order neurons (in e.g., ARC, LHA) able to negatively impact on energy balance.

Endorphins

The peptide β-endorphin is also derived from POMC and mainly expressed in the hypothalamus. Dynorphin-A is another endorphin with opioid-like effects. In male rats voluntary exercise under food-restricted conditions elevated β-endorphin in the ARC and dynorphin-A in the supraoptic hypothalamic nucleus following 2-deoxy-D-glucose (2DG) stimulation (38). In addition, in the suprachiasmatic nucleus dynorphin-A was increased (37). Female rats undergoing the ABA paradigm did not display alterations of hypothalamic β-endorphin concentration compared to controls (141). These observations in ABA resemble data of increased opioid activity in humans with AN (142); thus, further studies on the role of opioid signaling in AN are warranted.

Central Orexin and Leptin

Female rats with a proactive stress-coping style to prenatal stress, assessed by the defensive burying test, displayed elevated hypothalamic mRNA orexin expression, higher levels of DNA methylation of the orexin gene as well as reduced leptin levels while their ghrelin levels were increased during ABA (48). In male ABA rats, chronic leptin infusion using minipumps, concomitantly to the initiation of food restriction for 7 days, suppressed the 300% increase of baseline physical activity observed in restrictively fed vehicle animals (12). Also, chronic subcutaneous (sc) application of leptin after initiation (6 days) of semi-starvation-induced hyperactivity abolished this alteration (12). Chronic leptin treatment (icv via osmotic minipump, 4 μg/d) in female ABA rats decreased running wheel activity, food intake, and increased energy expenditure by thermogenesis (65). Similarly, in female rats undergoing ABA, acute central leptin injections into the lateral ventricle and microinjection of leptin into the ventral tegmental area at day 4 diminished running wheel activity (79), pointing toward a role of decreased leptin signaling in hyperactivity.

Corticotropin-Releasing Factor (CRF)

CRF, expressed in the PVN and stimulating adrenocorticotropic hormone (ACTH) secretion, is a hallmark regulator of the HPA

axis and thus involved not only in the stress response but also energy metabolism (137). Seven-day running wheel access increased CRF mRNA expression in the DMH, but not in the PVN, of male rats (143). Similarly, ABA in female Sprague Dawley rats did not alter CRF mRNA concentrations in the PVN (86). Interestingly, female rats that developed ABA during adolescence presented increased anxiety-like behavior associated with increased expression levels of CRF mRNA in the PVN and the central nucleus of the amygdala in adulthood, whereas food restriction alone did not induce these changes (144). Another study showed that ABA elevated c-Fos in CRF positive neurons of the PVN and DMH (76). The effects of wheel running on meal size could be reversed by icv injection of the CRF antagonist α-helical-$CRF_{(9-41)}$, additionally increasing DMH CRF mRNA expression (143), pointing toward a crucial role of CRF in the development of ABA. In another study, icv injection of the competitive CRF antagonist SHU9119 had no ameliorating effect on ABA, while treatment with the inverse agonist $AgRP_{(83-132)}$ did (64). Therefore, the role of CRF in the development and maintenance of ABA needs further investigation.

Nesfatin-1

Nesfatin-1 is an anorexigenic neuropeptide found predominantly in the hypothalamus (and peripherally in the gastric mucosa) also involved in the modulation of gastrointestinal functions (145). Phenotyping studies in female rats showed that following a 7-day wheel acclimatization +14 day food restriction protocol with or without a running wheel, the number of activated nesfatin-1 immunoreactive cells was increased in the PVN, ARC, DMH, dorsal raphe nucleus and the rostral raphe pallidus compared to *ad libitum* fed and activity (running wheel) controls (75). Also, food restricted rats showed a trend toward an increase of NUCB2/nesfatin-1 (most antibodies do not distinguish between full length NUCB2 and nesfatin-1) protein expression in the PVN, ARC, LC, and DMH, while in rats with access to the running wheel only no altered expression of nesfatin-1 was observed compared to *ad libitum* fed control rats (75). The differences between ABA and food restricted rats indicate central alterations independent of a simple body weight reduction. Taken together, alterations in expression patterns can be observed in motor and higher food intake circuitries due to ABA, likely underlying/contributing to the effects on food intake and locomotor activity. These activity patterns resemble those observed in humans with AN, e.g., altered cortical processing of high and low-calorie food pictures compared to healthy controls has been described (146).

Serotonin

Serotonin (5-HT) as one of the most important central neurotransmitters is widely expressed in the human brain. It is thus involved in various regulatory processes, modulating—among others—mood, anxiety, aggression, and hunger (147). The serotonin receptors can be subdivided into seven subtypes: 5-HT_1-5-HT_7, all of which are G-protein coupled receptors with the exception of 5-HT_3, which is an ion channel. Serotonin's appetite-suppressing effect is mainly mediated by 5-HT_{1B}, 5-HT_{2C} and 5-HT_6 receptors in the ARC and PVN (148). In humans, evidence showing a robust effect of antidepressants on body weight gain in AN is limited so far (149). However, the combination of selective serotonin re-uptake inhibitors (SSRI) with psychotherapy can exert positive effects on anxiety, depression, and compulsive thoughts (150).

Dietary restriction inducing hyperactivity reduced central, especially hypothalamic, serotonin signaling (151). A reduction of serotonin concentration has also been observed in AN patients (152). Daily administration of fluvoxamine (7 d, orally), a SSRI, suppressed the increase in running activity after feeding without affecting body weight loss or food intake in ABA rats (109). In female ABA mice, chronic oral treatment with fluoxetine, another SSRI, elevated food intake, and reduced activity without any effects on survival (69). Similarly, female ABA rats, treated ip with fluoxetine for 5 weeks, showed an increased food intake, displayed decreased running activity and lost less weight compared to saline treated rats (83).

Agonists with high affinity for the 5-HT_{1C} receptor [located in cortical and subcortical neurons of the hippocampus, thalamus, and monoaminergic cell groups (153)] blocked semi-starvation-induced increased running wheel activity in rats (108). Daily sc injections of 8-OH-DPAT, an agonist of the 5-HT_{1A} receptor (found pre- and postsynaptically, ubiquitously located, and stimulating cAMP formation in the respective neurons), administered 40 min before feeding for 10 days prevented hyperactivity, subsequently reducing body weight loss in female ABA rats (46). In contrast, hyperactivity, induced by restricted access to food in female rats, was enhanced by acute sc injection of 8-OH-DPAT (151), pointing toward different effects of 5-HT_{1A} and 5-HT_7 activation in ABA and food-restricted animals.

Fenfluramine (continuously infused sc during 1 week), an appetite suppressant activating 5-HT_{2C} receptors and releasing serotonin resulting in increased extracellular serotonin levels, did not affect food intake, wheel running, body weight, hypothermia or HPA axis activity, while inducing hypodipsia, elevated plasma osmolality, and arginine-vasopressin expression levels in the hypothalamus (62). In another study, ABA rats chronically sc infused with fenfluramine using mini-pumps had a greater susceptibility to ABA due to a reduction of food intake (43). Similarly, fenfluramine (acute ip injection) administered 1.5 h prior to the daily 2-h period of food access in female ABA rats induced an accelerated weight loss (45). Female ABA rats treated with parachlorophenylalanine, an irreversible tryptophan hydroxylase inhibitor depleting serotonin, showed decreased food intake and increased running activity resulting in increased body weight loss (83), suggesting an inverse correlation between central serotonergic activity and vulnerability to develop ABA. Taken together, these observations indicate that signs of ABA (weight loss, hyperactivity) can be intensified by increasing peripheral 5-HT signaling, while centrally a reduction exerted a similar effect.

Fluoxetine, another SSRI, was shown to exert various effects on ABA animals. In detail, it decreased dynorphin-A content under ABA conditions (37), induced a pathological elevation of vasopressin in the suprachiasmatic hypothalamus, while a physiological increase in plasma vasopressin was observed (37, 42). Additionally, it reduced oxytocin secretion in ABA animals,

while increasing oxytocin in control animals (37, 42). Lastly, fluoxetine had no effect on parameters like blood glucose, plasma insulin or insulin-like growth factor 2 (IGF-2) concentrations in ABA (154).

Alterations of Central Structures and Transmitters Involved in Reward-Motivated Learning

Eating behavior is strongly linked with the reward system. Also, eating disorders may be interpreted as reward-dependent since e.g., reducing eating is perceived as rewarding in AN (155). Thus, various studies examined central areas involved in reward in the context of ABA.

Examining brain activity using a micro PET-CT, increased cerebral 18F-fluorodeoxyglucose uptake was observed in male ABA rats in the mediodorsal thalamus [playing a role in memory and cognition (156) and body weight regulation (157)], ventral pontine nuclei and cerebellum [modulates motor activity, involved in classical conditioning (158)] compared to active and inactive *ad libitum* fed controls (71). On the other hand, ABA was associated with a reduced uptake in the left rhinal and bilateral insular cortex [responsible for gustatory and viscerosensory processing; (127)] as well as bilateral ventral striatum [involved in processing of motivation, aversion and reward, lesions results in increased food intake and weight gain; (127)] compared to control animals without food restriction and without access to running wheels (71). Noteworthy, brain metabolism in the cingulate [processing reward by linking actions and emotions resulting in learning (159)] and surrounding motor and somatosensory cortex was positively correlated with body weight (71). These data suggest a significant role of central structure involved in reward signaling in the development and/or maintenance of ABA.

Similarly, electrical stimulation or an electrolytic lesion of the mediodorsal thalamus did not affect signs of ABA in female rats that already developed ABA, while a preventive lesion selectively decreased hyperactivity in ABA later on (70). Future studies using electrical stimulation should include additional brain areas to possibly affect features of AN.

Dopamine

Dopamine expressed in the ventral tegmental area (VTA) as part of the mesocorticolimbic dopamine system is a key regulator of the reward and motivation system (160). The dopamine receptors are categorized into two families; the D_1 and D_5 receptors belong to the D_1-like family, which are G-protein coupled, increase cAMP and can be mainly found post-synaptically, while D_2, D_3, and D_4 receptors represent the D_2-like family inhibiting cAMP formation and are located both pre- and post-synaptically. In brief, stimulation of D_1 receptors results in dilatation of cerebral vessels. D_2 receptors modulate motor activity similar to D_4 receptors, while D_3-receptors, located in the limbic system and cortex, are involved in cognition (161).

Restricted food access elevated mRNA expression levels of neuronal cell adhesion molecule 1 (NCAM1), involved in the formation and modulation of synaptic contacts in the VTA of Balb/cJ female mice (67).Wheel running alone elevated mRNA expression of the growth factor, brain-derived neutrotrophic (BDNF) in the VTA. When both conditions were combined, no effects on BDNF or NCAM1 mRNA expression within the mesocorticolimbic pathway could be observed (67). Female A/J inbred mice with typical signs of ABA showed elevated dopamine D_2 receptor expression in the caudate putamen (59). In female ABA rats, dopamine release in the nucleus accumbens was found not to be elevated during the initiation of food-anticipatory behavior but it was increased during food intake; additionally, serotonin levels were decreased and circadian activity was diminished (78). An elevation of dopamine levels has also been observed in AN patients (162).

As mentioned above, an effect of antidepressants on significant body weight gain in patients with AN could not be detected so far (149). Among psychotropic drugs, olanzapine, a non-selective modulator of various neurotransmitter systems such as dopamine signaling, was the only medication found to accelerate weight gain and reduce mealtime anxiety in AN; however, inconsistent data exist (150). In an ABA model, during food-anticipatory activity (locomotor activity that occurrs 2 h before the availability of food), levels of dopamine and its metabolites in the striatum and midbrain were upregulated (163). Consequently, treatment of ABA BALB/cJ mice with olanzapine (orally applied daily for 7 days) antagonizing dopamine and serotonin receptors increased survival by decreasing food-anticipatory activity (164). Similarly, ABA rats treated with olanzapine (sc daily for 7 days using an osmotic minipump) displayed a decreased running wheel activity rate, starvation-induced hypothermia and activation of the HPA axis, indicated by decreased levels of ACTH, corticosterone and adrenal weights without affecting food intake, while body weight loss was decreased and ABA development diminished (66). Daily oral treatment with the $D_{2/3}$ receptor antagonists eticlopride and amisulpride in ABA Balb/cJ female mice decreased weight loss and hypophagia, resulting in increased survival (68). Additionally, amisulpride reduced weight loss and hypophagia to a higher extent compared to olanzapine (68). Similarly, the D_3 receptor antagonist SB277011A or the D_2 receptor antagonist L-741,626 elevated survival (68). Also, application of chlorpromazine, another dopamine receptor antagonist, reduced hyperactivity, leading to a 75% decreased mortality (11). In male rats, chlorpromazine (intraperitoneally, ip) blocked food suppression after wheel running during the light phase in an acute model of ABA (165). ABA rats treated chronically with the non-selective dopaminergic D_1 and D_2 antagonist cis-flupentixol reduced body weight loss and increased food intake (77). The D_1 receptor antagonist SCH23390 as well as the D_2 receptor antagonist raclopride (acute ip injection) significantly decreased food-anticipatory activity compared to controls, while co-administration of both showed an additional effect on the reduction of food-anticipatory activity (163). Noteworthy, dopamine D_1-like antagonists such as SCH23390 did not alter survival (68). The hypothesis that antagonizing dopamine signaling has beneficial effects by increasing food intake and body weight and reducing hyperactivity in ABA should be substantiated in future studies.

Excitation of the reward pathway by means of a dual viral strategy involving retrograde transport of Cre to the ventral tegmental area and coincident injection of DREADD receptors inducing recruitment of a large proportion of VTA-NAc dopaminergic projections decelerated establishment of ABA in female rats by increasing food intake, food-anticipatory activity and reducing weight loss (93). In female rats a direct correlation between the intensity of activity and the severity of withdrawal symptoms, assessed following sc naloxone injection, was observed; thus, ABA rats displayed the most withdrawal symptoms compared to restrictively fed, *ad libitum* fed and active controls (96). Taken together, these results indicate an alteration of the reward system during ABA in line with abnormal reward circuitries described in AN (166). Options to modulate the reward system modulating the severity of ABA should be examined further.

Alterations of Hippocampal Structures and Transmitters

The hippocampus is responsible for transmitting memory content from short to long-term memory (167). Restricted feeding for 14 days increased the transcripts of the growth factor BDNF in the hippocampus of Balb/cJ female mice (67), suggesting a putative role of the hippocampus in ABA. In contrast, female A/J inbred mice undergoing the standard ABA protocol showed decreased BDNF expression in the hippocampus (59). Similar to these inconsistent data of BDNF alterations in ABA, studies on BDNF levels in AN have shown variable results (168).

In adolescent female Sprague Dawley rats, cell proliferation in the dentate gyrus and hilus region, but not in the subgranular zone and in the surrounding dorsal hippocampus and in the corpus callosum, was reduced after 3 days of ABA with a positive correlation between cell proliferation and body weight/food intake (29), indicating an effect of ABA in adolescents rather on gliogenesis than on neurogenesis. Female ABA rats showed reduced total dendritic length and dendritic branches in the stratum radiatum of the dorsal hippocampus, responsible for spatial learning and cognition; in contrast, branching in stratum radiatum of the ventral hippocampus mediating anxiety was elevated in ABA (24). Exercise mainly affected stratum radiatum, while food restriction influenced the stratum lacunosum moleculare in the dorsal and ventral regions (24), pointing toward pathway-specific alterations in the hippocampus due to ABA. ABA in female adolescent Sprague Dawley rats increased branching of ventral hippocampal pyramidal cells, while the same protocol in adulthood decreased branching of ventral hippocampal pyramidal cells without any effects on dendritic branching (25). The proportion of mature spines on dendrites was also altered due to ABA: in adolescent female ABA animals it resembled adult control animals since control animals doubled branching from adolescence to adulthood (25). The results underline the age-dependent vulnerability of hippocampus plasticity to ABA. Noteworthy, relapse of ABA decreased branching (25). Thus, the hippocampus is an important structure implicated in the development of ABA.

Hippocampal Gamma-Aminobutyric Acid (GABA)

In the hippocampus of female ABA rats a 6-fold increase of α4 subunits of α4βδ GABA receptors and a 130% increase of δ subunits of α4βδ GABA receptors, sufficient to increase tonic hippocampal inhibition, was observed compared to age-matched control females (23). GABAergic inhibition in the hippocampus strongly induces anxiety and additionally regulates plasticity (169). Similarly to the findings in the hippocampus, in the amygdala of female pubertal rats under ABA conditions an increase of membranous α4 subunits near excitatory synapses on dendritic shafts in the caudal basal amygdala accompanied by intracellular elevation of α4 subunits was observed, indicating a disinhibition of the excitability of the amygdala (26). Hyperactivity during food restriction in ABA adolescent female rats negatively correlated with α4βδ GABA receptor levels visible within 2 days of food restriction (27), suggesting a protection against ABA by inhibition of α4βδ-GABAARs in spines of CA1 pyramidal neurons suppressing physical activity.

A negative correlation was described between α4 subunit concentration at spines of pyramidal cells of the hippocampal CA1 with severity of ABA, measured as food restriction-elicited running activity during ABA (170), suggesting a protective role of α4 subunits counterbalancing the ABA-induced excitability of CA1 pyramidal neurons.

Contact lengths of axo-somatic contacts made by GABAergic axon terminals onto layer 5 pyramidal neurons were increased by 40% in female ABA mice; thus, the proportion of L5P perikaryal plasma membrane contacted by GABAergic terminals was elevated accordingly (171). Additionally, in female ABA mice a negative correlation was observed between contact length in the anterior cingulate cortex and overall wheel activity after food restriction and between contact length in the prelimbic cortex and wheel running especially during food availability in the restriction phase (171). Adolescent female C57BL/6 mice that developed ABA with food access during the first 2 h of the dark cycle all survived; when re-exposed to the same conditions after recovery for 7–11 days only some were vulnerable to ABA with those being vulnerable displaying a reduced GABAergic innervation on cell bodies and dendrites in CA1 pyramidal cells compared to resilient mice (32). In summary, this underlines that GABAergic innervation of hippocampal structures contributes to the protection of animals against ABA.

In rats, chlordiazepoxide (acute ip injection), a benzodiazepine, suppressed the decrease of food intake under conditions of ABA (172).

Hippocampal N-Methyl-D-Aspartat (NMDA)

While GABA is the main inhibitory, glutamate is the major excitatory neurotransmitter in the brain (173). Hippocampal NMDA, as part of glutamate receptors and ion channel protein, is involved in modulating learning, memory processing, and feeding behavior (174). Using electron microscopy hippocampal synaptic NR2A-NMDA and NR2B-NMDA receptor levels were observed to be increased in female ABA rats (31). In those animals, ABA severity positively correlated with synaptic NR2B-NMDA receptor levels (31). In rodents resilient to ABA

that did not develop hyperactivity, reserve pools of NR2A-NMDA receptors in spine cytoplasm correlated with the suppressed physical activity (31). NR2A- and NR2B-NMDA receptors were related to spinous prevalence of an F-actin binding protein, drebrin, responsible for receptor insertion to and retention from synaptic membranes (31), indicating that increased NMDA receptor expression elevates the vulnerability to ABA. Noteworthy, anti-NMDA receptor encephalitis in humans, resulting in decreased receptor density, is also associated with abnormal eating behavior (175).

Subchronic treatment with agmatine (ip, for 10 days), an endogenous ligand of imidazoline and α2-adrenergic receptors that additionally selectively blocks the NMDA subclass of glutamate receptor channels, reduced hyperactivity, increased food intake and normalized body weight of female ABA rats, also decreasing corticosterone levels (106), probably resulting from restored body weight and increased food intake.

Clonidine, an α2-adrenergic receptor agonist, inducing sympatholytic effects such as a reduction of blood pressure (176) via negative feedback mechanisms was also tested under conditions of ABA. Chronic infusion of clonidine into the PVN of male ABA rats resulted in a dose-related increase in the susceptibility to ABA and a decrease in food intake; similarly, in heavy animals an increased susceptibility to ABA was observed after chronic infusion of clonidine into the PVN but without effect on food intake or wheel activity (41). Male rats receiving a continuous sc infusion of clonidine using osmotic minipumps and exposed to ABA showed an increase of food intake at a lower dose of clonidine and a stimulation of wheel activity at a higher dose, with no effects on weight loss (40), indicating that centrally applied clonidine increases the vulnerability to ABA, an effect mimicked by higher peripheral doses presumably crossing the blood-brain barrier.

Cannabinoids

A rich hippocampal expression of the type 1 cannabinoid (CB_1) receptor suggests an important role of cannabinoids in the hippocampal network and memory formation (177).

ABA conditions increased absolute CB_1 receptor binding using (18)F-MK-9470 in all cortical and subcortical brain areas in both sexes, which decreased again in the recovery phase (73). Elevation of relative CB_1 receptor binding was observed in the hippocampus, inferior colliculus and entorhinal cortex in female ABA rats, which also normalized with weight regain in the recovery phase (73), giving rise to impaired endocannabinoid transmission under conditions of ABA, a finding also reported in humans with AN (178).

In male C57/BL6 mice undergoing ABA daily ip application of the phytocannabinoid delta(9)-THC 30 min before the dark phase reduced survival but increased feeding in the animals which did survive, while the anandamide analog OMDM-2 stimulated food intake without sufficiently reversing weight loss (99). Subchronic ip THC treatment 30 min before the onset of the dark phase in female ABA rats transiently increased food intake and also affected running wheel activity (179). The higher dose also decreased body weight loss accompanied by reduced energy expenditure and lipolysis (179). When combined with high fat diet, THC had the same effects but to a greater extent (179). Daily ip injection for 6 days with the CB_1/CB_2 receptor agonist Δ9-tetrahydrocannabinol or the CB_1/CB_2 receptor agonist CP-55,940 decreased body weight loss, physical activity, and plasma corticosterone levels while increasing leptin signaling in female ABA rats (104). Noteworthy, treatment was initiated at the start of a second ABA protocol after rats already experienced one ABA and one recovery phase (104). Overall, the cannabinoid system is able to increase food intake also under conditions of ABA. Noteworthy, although the CB_1 receptor is highly expressed in the hippocampus and might mediate the effects mentioned above via the hippocampus, it should be kept in mind that this receptor is also found in the VTA and hypothalamus. It cannot be excluded that the orexigenic effects observed after CB_1 receptor agonist application are mediated via VTA and hypothalamus, a hypothesis which should be examined further.

Consequently, male rats that orally received the CB_1 receptor antagonist SR141716 over a period of 32 days prior to ABA starved faster, lost weight faster and increased the wheel running rate more rapidly compared to those without drug treatment (88). Rats with the same drug treatment but lacking a functional leptin receptor did not reach the starvation criterion of 25% body weight loss (88). Additionally, they displayed reduced wheel running as well as decreased levels of serotonin and its metabolic products in the hypothalamus and neural-reward areas including the nucleus accumbens compared to animals with the same dysfunctionality but without drug administration, suggesting an interaction between CB_1 and leptin receptor signaling also implicated in regulating energy balance (88).

Alterations in Widely Expressed Transmitters Involved in Hyperactivity

Histamine

Histamine is a widely expressed transmitter signaling—among others—via the Gq-protein coupled H_1 receptor resulting in a calcium release, involved in the regulation of vomiting, sleep and adrenalin secretion and via the mostly pre-synaptically located H_3-autoreceptor modulating release of acetylcholine, noradrenalin, and serotonin thus being involved in the regulation of hunger, body temperature, and blood pressure (180).

Male ABA rats exhibited decreased H_1 receptor binding in the cortex, diencephalon and hippocampus; in contrast, decreased H_3 receptor binding in cortex and diencephalon due to an acute forced swim test was normalized under conditions of ABA (90). ABA gradually increased central (cortical, diencephalic, and hippocampal) histamine levels and icv administration of additional histamine reduced wheel running activity (90), giving rise to the speculation that the upregulation of central histamine represents a compensatory attempt to reduce hyperactivity. Similarly, humans with AN also showed increased H_1 receptor binding in the amygdala and lentiform nucleus (181).

Pyrilamine (acute ip injection), an H_1 receptor antagonist, reduced locomotor activity during the dark period in *ad libitum* fed mice, without exerting effects on food-anticipatory activity under ABA conditions in mice (163).

Noradrenaline

Noradrenaline, greatly expressed in the LC, affects various functions including sleep-wake regulation, arousal, attention, and memory (182). ABA in female rats activated LC neurons compared to *ad libitum* fed rats as assessed using the neuronal marker c-Fos (74).

Excessive exercise due to food restriction for 4 days in female Sprague Dawley rats reduced cerebellar noradrenergic fiber length, while exercise in general decreased inter-varicosity interval length and increased varicosity density along noradrenergic fibers (30). Rats that did not respond to the ABA protocol, namely rats that suppressed food restriction-evoked excessive exercise, displayed shortened inter-varicosity intervals resulting in blunting of body weight loss (30). Whether changes in fiber length and varicosity density are cause or result of ABA should be evaluated in further research.

Increased locomotion as a response to food restriction is still a barely understood phenomenon in AN. Therefore, further investigations of the involvement of histamine and noradrenaline might lead to possible strategies to counteract these changes.

Alterations in Anxiety and Anhedonia and Respective Central Regulatory Areas

ABA in adolescent female mice decreased anxiety as assessed using the elevated plus maze test with a negative correlation between the time in open arms and food restriction-induced wheel activity during the following 24 h; thus, mice displaying high anxiety were hyperactive (183). Using the open field and plus maze tests it was shown that female rats that underwent ABA during adolescence displayed an increased anxiety-like behavior in adulthood, whereas animals subjected to food restriction alone did not (144). The ABA protocol performed twice with 7 days in between in adolescent female rats induced an increased long-term anxiety-like behavior in adulthood as assessed by the elevated plus maze test (97). Animals showed reduced ERbeta signaling in the amygdala using quantitative real-time PCR; however, ovariectomy was unable to prevent long-term behavioral changes (97). Strikingly, another study showed no effect of ABA on anxiety-like behavior as assessed using the elevated plus maze and open field test (49). Refeeding without wheel access after ABA decreased horizontal activity and exploratory horizontal behavior (13).

Male BDNF-Val66Met knock-in mice (BDNF Met/Met) displayed a decreased activity-dependent BDNF secretion and increased anxiety-like behavior (34). Strikingly, under ABA conditions wildtype mice did not differ from BDNF Met/Met mice regarding anxiety and lost GABAergic innervation along distal dendrites in the hippocampal CA1 region and medial prefrontal cortex (34). BDNF Met/Met mice showed reduced food restriction-evoked hyperactivity (34) leading to the hypothesis of blunted vulnerability to ABA.

Only one quarter of female ABA Sprague Dawley rats exhibit transient anhedonia (enduring food restriction and hyperactivity, disappearing during weight restoration) as assessed using the sucrose preference paradigm (94). Noteworthy, exposure to a running wheel correlated with an aversion to sweetened water, and high levels of hyperactivity before food restriction correlated with high susceptibility to body weight loss in ABA (94). Additionally, food-anticipatory activity was related to subsequent food intake only in body weight loss-resistant rats (94). No effect of ABA on unconditioned lick responses to sucrose or quinine or on preference for a diet high in fat could be observed in female rats, indicating no alterations of taste responsivity in ABA rats (49).

Since anxiety and other behavioral changes such as affective, anxiety, obsessive-compulsive, and substance abuse disorders can be observed in AN as well (3), the model also helps to assess these comorbid conditions.

Alterations in Behavior

When examining operant responding for food reinforcers in male albino ABA rats, each nose-poke response was reinforced by a food pellet during the feeding phase resulting in a steeper decrease in nose-poke response in ABA (84), indicating impaired tolerant learning. ABA also induced an impairment in reversal learning at low weight assessed using the attentional set-shifting test with normalization following weight restoration in female rats (82). ABA in adolescent female rats reduced performance during the novel object recognition task but not in the novel place recognition task or the Barnes maze (49), suggesting impaired contextual but not spatial learning. The underlying central mechanisms warrant further investigation in order to potentially improve treatment of cognitive deficiency in AN.

After experiencing ABA during adolescence until a 25% reduction of baseline body weight and following 10 days of body weight regain, the acquisition of an aversion to sucrose was accelerated and reinforced compared to female control rats (50). Likewise, the extinction process was altered in post-ABA female rats with a significant slowing of extinction in the one bottle test where sucrose is presented for 5 min without following injection (50). These data might provide an underlying mechanism contributing to the high relapse rate in AN (184).

Alterations of Peripheral Hormones

Oxytocin

In food deprived animals, oxytocin within the thymus was decreased, likely due to reduced thymus gland weights also observed under conditions of ABA (86). Similarly, in humans serum oxytocin levels were reported to be decreased in AN compared to healthy controls (185).

Corticosteroids

ABA in female Sprague Dawley rats led to higher baseline corticosterone levels (48). Similarly, male and female ABA rats displayed elevated circulating corticosterone concentrations associated with higher relative adrenal gland weights, (86). Interestingly, female rats that developed ABA during adolescence presented increased anxiety-like behavior associated with elevated plasma corticosterone (144). Wheel running induced by hypophagia was absent in adrenalectomized male Lewis rats, a finding reversible by corticosterone replacement (186). Similarly, pre-prandial hyperactivity was diminished by adrenalectomy and restored by acute corticosterone injection (186). Also, in humans

with AN, cortisol concentrations were elevated (187), possibly contributing to hyperactivity. The underlying mechanism of corticosterone elevation in AN should be examined in more detail.

Ghrelin

Plasma ghrelin levels in female mice were correlated with food-anticipatory behavior observed as running activity under conditions of ABA; conversely, female ghrelin receptor (GHS-R1a) knockout mice did not anticipate food (presented as percentage of total running wheel activity) (81). Similar effects were observed in ABA mice treated either acutely icv or chronically peripherally with a GHS-R_{1a} antagonist that did not show alterations in food intake (81). Additionally, food restriction in ABA C57BL/6 male mice increased preproghrelin mRNA-expressing cells in the stomach proportionally to body weight loss (16). Single daily ip injection of ghrelin and ghrelin combined with IgG from obese, but not lean mice, prevented ABA in male C57BL/6 mice by decreasing physical activity during the feeding period without diminishing body weight loss and altering food-anticipatory activity (18). These inconsistent data, showing decreased running activity under ghrelin receptor knockout conditions as well due to exogenous ghrelin application, could be a result of different routes of application or protocols used. In summary, these results suggest that ghrelin suppression might be an interesting target to tackle hyperactivity in AN.

A study in C57BL/6J and DBA/2J inbred mouse lines showed that food reduction leads to hypoleptinemia. The comparison of both strains additionally showed that C57BL/6J mice reduced wheel activity due to food restriction, while DBA/2J mice displayed hyperactivity correlating with a stronger plasma leptin decline, indicating that dynamic changes of plasma leptin have a greater impact on the development of ABA than a simple reduction of leptin levels (58).

In male Sprague Dawley rats the ABA paradigm was shown to significantly reduce circulating leptin levels and increase ghrelin levels (100). Especially in visceral and gonadal fat leptin was absent (100). Interestingly, expression of ghrelin and leptin (LEPR) receptors was tissue-specifically altered in ABA, with increased GHS-R1a and LEPR expression in oxidative-soleus type of muscle compared to the glycolytic-gastrocnemius type (100). Additionally, GHS-R1a expression in visceral and subcutaneous fat was stimulated by ABA, while the active long form of LEPR was only expressed in subcutaneous fat (100). Disturbed regulation of leptin and ghrelin as seen in ABA rats has also been detected in AN patients (188, 189).

Female Reproductive Hormones

Restrictedly-fed female rats with access to wheels losing 25% of body weight in 8 days developed a disruption in the estrous cycle, an alteration restored after weight gain (47). Interestingly, while hypoactivity developing during recovery from ABA disappeared after resumption of estrous cycle, hyperphagia persisted but was limited to nonestrous phases (47). The ABA paradigm repeated twice in adolescent female rats with a 1-week interval in between induced reduced estrogen receptor (ER) beta signaling in the amygdala accompanied by anxiety-like behavior, long-term behavioral changes that could not be prevented by ovariectomy (97). In humans one of the main characteristics of AN is the reduction in estradiol (E2) levels resulting in secondary amenorrhea (190); consequently, this alteration should be mimicked by and further investigated in ABA.

Allopregnanolone, a metabolite of progesterone, sc administered during the 2nd food restriction period had no effect on wheel running activity in mice sensitive to ABA. In contrast, in ABA-resistant female C57BL6 mice it induced increased running activity compared to a resistant group receiving vehicle. Resistance was reflected by a reduction of wheel running in the course of ABA (170).

Alterations of Glucose Homeostasis and Energy Metabolism

In male ABA rats blood glucose levels and plasma insulin concentrations were decreased compared to *ad libitum* fed or exercising controls; however, peripheral IGF-2 concentrations were elevated (154). Similarly, in male Sprague Dawley rats ABA was shown to significantly reduce fat mass and increase insulin sensitivity (100). Also, female Sprague Dawley rats showed lower insulin levels (48). In male rats subjected to ABA, 2DG led to a reduction of food intake, a finding similarly observed in weight-matched controls suggesting a general effect of weight loss (39). Since hypoglycemia can be a life-threatening consequence in AN (191), the underlying mechanisms should be further examined.

ABA lowered body weight accompanied by an increase in lean/fat mass ratio and fat oxidation in male C57BL/6 mice (13). Refeeding with wheel access after the ABA protocol restored fat free mass (13). In male Wistar rats, wheel running activity reduced malondialdehyde, a degradation product of polyunsaturated fatty acids and a marker of oxidative stress, while food restriction decreased malondialdehyde plasma levels along with antioxidant capacity in liver and catalase activity in the gastrocnemius muscle (91). Additionally, the combination of both, food restriction and access to running wheel, elevated total antioxidant plasma levels but also reduced antioxidant parameters in the liver and plasma malondialdehyde levels compared to controls (91), presumably resulting from a reduced need of antioxidant activity in the liver associated with a higher plasma antioxidant capacity. Additionally, during refeeding after development of ABA, female rats displayed a lower resting energy expenditure and total energy expenditure resulting in higher weight gain, although energy intake was lower compared to controls (95). Although controls and ABA rats maintained similar body weights, lipid accumulation in visceral adipose tissue was reduced, while liver fat accumulation was increased in post-ABA rats, probably caused by overfeeding with carbohydrates (95). Therefore, the ABA model can also be used to study the consequences of weight restoration in AN.

Interestingly, ABA mice exhibited an activation of autophagy as assessed by increased dynamin-1 and LC3II/LC3I ratio (21). This was observed in the soleus muscle of ABA mice associated with reduced protein synthesis, while in the anterior tibialis no alterations were

observed. Compared to controls and restrictedly fed mice, C57BL/6 ABA mice displayed a reduction in dihydrolipoyl dehydrogenase and 3-mercaptopyruvate sulfurtransferase and other mitochondrial proteins implicated in energy metabolism (192).

Gastrointestinal Alterations

Comparing restrictively fed male C57Bl/6J ABA mice with mice on a restricted feeding schedule ABA mice had a thinner muscular layer and decreased claudin-1 expression in the colon associated with increased colonic permeability without differences in occludin expression or jejunal paracellular permeability after 17 days (17). Refeeding after ABA for 5 days without wheel access elevated colonic permeability, indicated by increased FITC-dextran flux compared to levels during ABA. Refeeding with wheel access increased muscle kynurenine conversion into kynurenic acid in male mice. Conversion prevents kynurenine, producing oxygen radicals and neurotoxins, to cross the blood-brain barrier, thus protecting from stress-induced depression, indicating a benefit of physical activity after ABA (13). Female C57Bl/6J ABA mice showed elevated Toll-like receptor 4 (TLR4) mRNA expression on colonic epithelial cells and intestinal macrophages, thus elevating downstream mucosal cytokine production. Simultaneously, hypothalamic interleukin-1β, interleukin-$1R_1$, and interleukin-1 receptor-associated kinase 4 as well as plasma corticosterone levels were elevated (19). TLR4-deficient mice displayed a higher mortality rate in response to the ABA protocol (19), suggesting a dual effect of TLR4 in ABA: a key role in the early colonic inflammation and a protective effect, since its absence can be fatal. Noteworthy, ABA in female mice induced an alteration in the colonic mucosal proteome, especially proteins implicated in energy metabolism (192). The mammalian target of rapamycin (mTOR) pathway was decreased inhibiting protein synthesis (puromycin incorporation) and activating autophagy (192), giving rise to an alteration of colonic mucosal proteome due to the downregulation of energy metabolism.

ABA in female rats activated neurons of the NTS [involved in the regulation of gastrointestinal motility (193)] compared to *ad libitum* fed rats as assessed using the neuronal marker c-Fos (74). In female ABA C57BL/6 mice, gastric emptying was delayed compared to controls without food restriction and without wheel access; similarly, animals with limited food access had delayed gastric emptying (20). Food intake microstructure during the feeding period in ABA did not differ from female animals without access to a running wheel during food restriction (74).

In ABA mice, a downregulation of proteins of the antrum was observed, namely ACTA2, VCL, KRT19, KRT8, and DES, proteins implicated in the organization of muscle fiber as well as heat shock proteins STIP1, HSPD1, and HSPA8 (20). Noteworthy, increased levels of gastric oxidized proteins were detected in female ABA mice (20). A total of 52% of rats that achieved a 30% weight loss due to ABA displayed gastric lesions (44) independent of sex but possibly associated with activity stress. Noteworthy, also in patients with AN various gastrointestinal alterations have been described, including decreased gastric emptying, impaired motility, and permeability (194).

Alterations of the Immune System

Wheel running activity and food restriction increased spleen weight and blastogenic response to lipopolysaccharide in female mice (92). It also decreased serum corticosterone levels, while food restriction alone increased corticosterone levels; however, without a significant correlation between serum corticosterone and any immune system measure (spleen weight and blastogenic response to lipopolysaccharide) (92). Exercise alone had no effect on *in vivo* antibody response to sheep red blood cells and *in vitro* splenic responses to concanavalin A and phytohemagglutinin (92). These observations suggest that exercise might prevent undernutrition-induced immunodepression similar to observations made in AN patients (92).

OUTLOOK AND CONCLUSION

In summary, ABA induces alterations in different homeostatic systems of the body (**Figure 2**). In the central nervous system, ABA changes the activity pattern in the motor and higher food intake circuitries as well as the expression of neuropeptides including AgRP, NPY, POMC, and CART in first-order neurons along with NMDA receptor expression. Additionally, hypothalamic protein synthesis is increased. ABA also modulates the GABAergic innervation and integrity of hippocampal structures. The endocannabinoid transmission is affected by ABA as well, along with an increased opioid activity. In addition, abnormal reward signaling can be observed under conditions of ABA. Regarding hormonal alterations, ABA induced an elevation of vasopressin and reduction of oxytocin. Cortisol and ghrelin concentrations are also elevated, while leptin is reduced. Hormonal changes in the reproductive system disrupt the estous cycle in ABA. Hypoglycemia and hypoinsulinemia are accompanied by increased insulin sensitivity in ABA. Functionally, decreased gastric emptying, increased permeability and prevalence of gastric lesions as well as gut inflammation and alterations in the colonic mucosal proteome, especially proteins implicated in energy metabolism, are typical gastrointestinal alterations found in ABA. Noteworthy, the physical activity in ABA can prevent undernutrition-induced immunodepression.

It can be concluded that ABA in rodents shares various similarities with AN in humans. However, as depicted in **Tables 1A,B**, there is a great heterogeneity of the protocols used to induce activity-based anorexia. Thus, results are often difficult to compare, pointing toward a necessity to standardize the protocol using a specific methodology regarding feeding schedule, acclimatization and housing conditions in order to obtain comparative studies.

To successfully reach the weight loss criterion in ABA, the following methodological details should be regarded: animals with a low body weight should be used, a period of wheel adaptation should be included and food restriction prior to the protocol should be omitted since ABA develops more likely when wheel running interferes with adaptation to the feeding schedule (which is avoided when the feeding schedule is introduced before the running wheel). Additionally, a feeding schedule for <2 h in rats with standard chow and *ad libitum* access

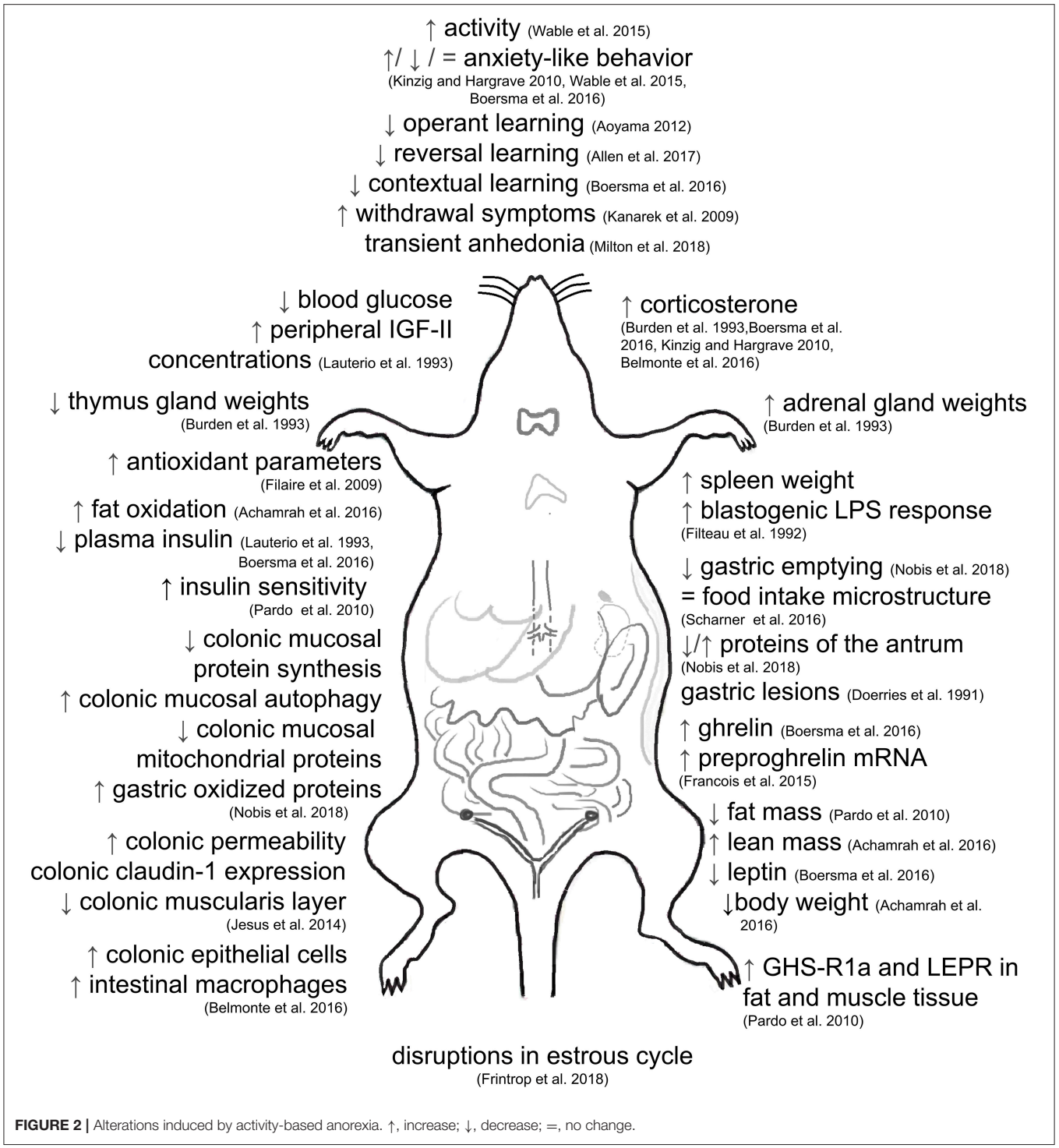

FIGURE 2 | Alterations induced by activity-based anorexia. ↑, increase; ↓, decrease; =, no change.

to water is necessary to induce hyperactivity and weight loss. Wheel access is crucial especially before food intake. Standard temperature should be guaranteed and isolation should be prevented in ABA, since it extends ABA duration by reducing hyperactivity (54).

Noteworthy, the ABA model has also several limitations. First, ABA only mimics some individuals with AN and not those without hyperactivity, since it combines food restriction and hyperactivity. Although hyperactivity is common in many individuals with AN, it is not present in all cases and it is not

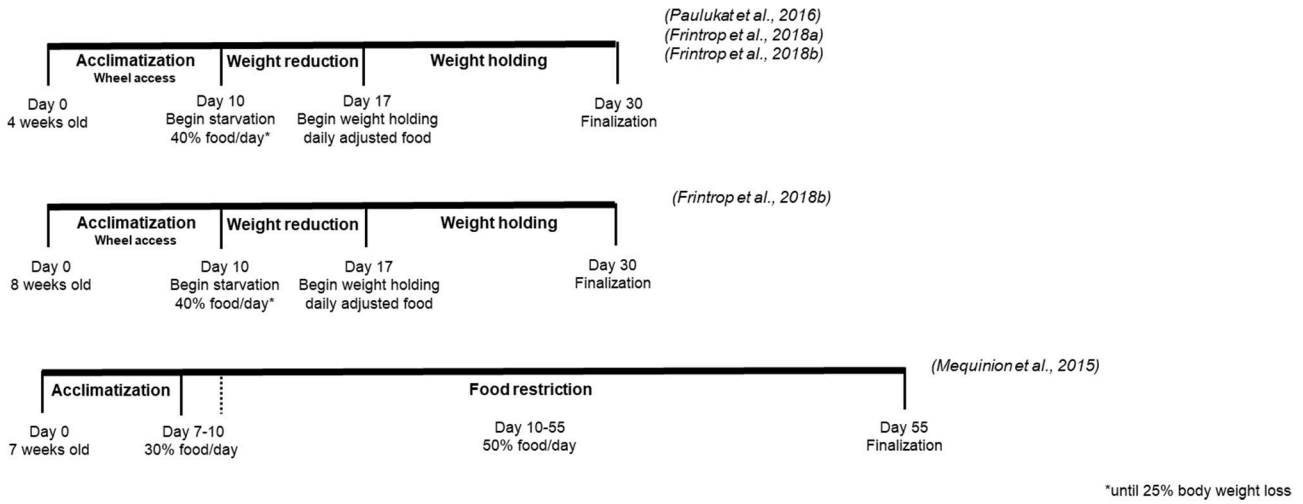

FIGURE 3 | Comparison of different chronic activity-based anorexia protocols.

a diagnostic criterion. Thus, ABA is no exact replication of the human disease. Second, when animals are placed in conditions with restricted food access and unlimited wheel access as in the present model, there are subgroups of animals who will not engage in running at all; these animals thus preserve body weight and can be maintained on restricted food access for a long time, whereas other animals will fail to consume sufficient food during the period of food access and will run themselves to death in <1 week (28). Some research groups exclude animals that fail to develop ABA; however, these animals that appear resistant to ABA could serve as a control group. Another limitation is the predominant use of male rodents in the ABA model although the majority of AN patients is female. Lastly, also this systematic review has limitations. Although the data search was performed in three different databases, it cannot be excluded that other relevant publications could not be identified and included. Similarly, a keyword-based search also has flaws, since sometimes keywords are omitted in publications. Moreover, articles in other languages than English were not taken into consideration. Lastly, for reasons of length and readability, not all results of the included publications could be shown.

Since AN is a disease enduring in most cases over several years and has a high relapse rate, in the last years few studies focused on the establishment of a chronic ABA model in order to mimic the pathological alterations in AN more closely (**Figure 3**). Comparing two protocols, one short-term over 15 days and another over 55 days, in the latter anticipatory hyperactivity was diminished over the course of the protocol, inducing also a reduction of lean mass and body fat as well as reduction of fat oxidation, preferential use of glucose to compensate for the chronic energy imbalance, decrease of leptin levels, increase of corticosterone, and ghrelin concentrations and a disruption of the estrous cycle (14). Wheel access did not prevent loss in bone mineral content due to food restriction and only the long-term protocol induced bone parameters similar to those observed in AN patients (14). Female adolescent rats that lost 20% of body weight due to ABA and subsequently exposed to an additional 2-week weight holding phase displayed a significantly disrupted menstrual cycle and E2 reduction compared to rats whose menstrual cycle was assessed just after the 20% body weight loss (195). Similarly, chronic starvation by food restriction to 40% of the baseline food intake and 24 h/day running wheel access until a 20–25% weight reduction followed by weight holding due to individual food restriction, resulted in a loss of the estrous cycle in all animals with 25% body weight loss, while acute ABA disrupted estrous cycle only in 58% of rats (123). In addition, due to the chronic ABA protocol in female rats an impaired memory function was observed with a correlation between E2 reduction and memory loss (195), possibly giving rise to E2 substitution as therapeutic attempt to treat cognitive deficits in AN. Chronic ABA also reduced the volumes of the cerebral cortex and corpus callosum, Glial fibrillary acidic protein positive astrocytes in these regions, total astrocyte-covered area and astrocyte mRNA expression (196), alterations that likely contribute to the neuropsychological deficits observed in AN. Lastly, comparing different ages, 4-week old rats displayed increased hyperactivity and amenorrhea compared to 8-week old animals (123), indicating that younger animals are more vulnerable to chronic ABA.

However, the chronic ABA models described above used a method of food restriction that includes offering a certain amount of food per day, which is different from the ABA model as stated in the introduction. In the standard model the animal is provided with an unlimited access to food for a limited period of time and has to choose between food intake and running wheel. In the amount-restricted model, animals will typically consume all of the food provided, a difference that should be kept in mind when comparing results. It might be useful to establish a chronic model with time restricted food access. Lastly, only a chronic protocol can induce robust alterations of the menstrual cycle and bone parameter, features also observed in AN. In the chronic protocol,

female rodents seem more suited since they endure a longer period without reaching the weight loss criterion (44, 120), while males showed a higher mortality rate due to ABA (13, 15). A chronic model including 4-week old rats losing 25% of body weight seems to most reliably display (chronic) AN characteristics.

AUTHOR CONTRIBUTIONS

MS wrote the first draft of the paper. AS thoroughly reviewed the manuscript. Both authors finalized the manuscript.

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Eating Behavior and the Evolutionary Perspective on Anorexia Nervosa

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On the standard perspective, anorexia nervosa and other eating disorders are caused by genetically determined, neurochemically mediated mental illnesses. Standard treatment, cognitive behavioral therapy (CBT), targets cognitive processes thought to maintain the disorders. Effective neurochemically based treatments are not available and the rate of remission is ≤25% 1 year after CBT, with unknown outcomes in the long-term. With starvation as the major threat in biological history, the evolutionary perspective focuses on foraging for food and eating behavior. A neural network, including hypothalamic arcuate peptide-neurons, brainstem serotonin- and dopamine-neurons and their prefrontal cortical projections, mediates (rather than controls) the behavioral adaptations to variations in food availability; activation of the network is associated with opposing behavioral outcomes depending upon external variations. In the clinic, the control of eating behavior is therefore outsourced to a machine that provides feedback on how to eat. Hundreds of eating disorders patients have recovered by practicing eating; the rate of remission is 75% in on average 1 year of treatment, the rate of relapse is 10% over 5 years of follow-up and no patient has died. A two-parameter asymptotic exponential growth curve modeled the eating behavior of 17 healthy women but not that of 17 women with anorexia nervosa. When in remission, the eating behavior of the anorexic women approached that of the healthy women. It is suggested that the treatment of eating disorders should focus on eating behavior.

Keywords: evolution, anorexia, eating, hypothalamus, brainstem, prefrontal cortex, treatment, mathematical models

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INTRODUCTION

"Anorexia nervosa is a psychiatric disorder characterized by fear of weight gain and dangerously low body weight … mortality rate exceeds that of other psychiatric disorders … finding comprehensive brain-based models … has been difficult" (Frank et al., 2018). Thus start most accounts. But it was recently suggested that this standard perspective needs to be modified because the treatment of anorexia is at a standstill (Gutierrez and Birmingham, 2018). We will describe the standard perspective and its translation into clinical practice first and then we will describe the evolutionary perspective, with eating behavior in clinical practice.

THE STANDARD PERSPECTIVE

On the standard perspective, anorexia is caused by a pre-existing, neurochemically mediated, genetically determined mental disorder as outlined some time ago as: "We hypothesize that people

with anorexia nervosa have a trait-related *increase* in 5-HT neuronal transmission that occurs in the premorbid state and persists after recovery" (Kaye et al., 2003) and: "Childhood anxiety represents one important genetically mediated pathway toward the development of anorexia nervosa and bulimia nervosa" (Kaye et al., 2004). The perspective is similar today (Treasure et al., 2015).

Clinical Translation of Neurochemistry and Genetics

If anorexia is caused by an increase of 5-HT synthesis an inhibitor or an antagonist should be used, but paradoxically, indirect agonists are used, and although useful in patients with mental disorders (Locher et al., 2017), these drugs are not useful in patients with anorexia (Walsh et al., 2006; Zandian et al., 2007). But neither are other drugs, including neuroleptics, which are valuable for patients with mental disorders (Lieberman et al., 2005), useful in patients with eating disorders (Attia et al., 2019). This differential effectiveness of psychopharmacological intervention may be because the "mental disorders" of eating disorders differ from those of patients with mental disorders. Thus, a rating scale that dissociates anxiety from other mental disorders in patients with mental disorders did not dissociate these disorders in 358 patients with anorexia nervosa [PS and others, manuscript submitted for Gutierrez and Birmingham (2018)].

The discovery that mental disorders are not distinct categories but vary along continuous dimensions was made long ago (Fisher, 1918; Porter, 2018), emphasized not long ago (Borsboom et al., 2011) and recently re-discovered (Brainstorm Consortium, Anttila et al., 2018; Plana-Ripoll et al., 2019; Schork et al., 2019). Hence, attempts to find genotype-phenotype correlations among eating disorders and mental disorders have yielded inconsistent results (Borsboom et al., 2011). Translating these results into treatments for eating disorders will be difficult (Breithaupt et al., 2018). This approach, which was launched 20 years ago in other contexts, has been marginally successful (Joyner and Paneth, 2019).

The Standard Treatment

The standard treatment, cognitive behavioral therapy (CBT), assumes that eating disorders are maintained by cognitive processes. Even though CBT does not address the cause of eating disorders, it recognizes that the patients' problems start with dieting (Slade et al., 2018). Launched for bulimia nervosa in 1981 (Fairburn, 1981), CBT is now recommended in the treatment guidelines for all eating disorders throughout the world [e.g., (NICE, 2017)].

Rather few patients have been treated with CBT in randomized controlled trials (RCT) (Slade et al., 2018). With a dropout rate ≈30%, which is generally expected and included in the power calculations of RCTs (Zipfel et al., 2014), a rate of remission <50% and a rate of relapse ≥30% within 1 year, ≤25% of the patients remain in remission at this point in time (Södersten et al., 2017).

Many more patients have been treated with CBT in general practice. For example, out of 683 patients referred to primary care for the treatment of bulimia within the United Kingdom healthcare system, 135 completed the treatment but although they improved, these patients did not remit (Knott et al., 2015). In Sweden, 15,411 patients were similarly treated in years 2012–2017 with a rate of remission of 18.4% at one year follow-up (Birgegård and Norring, 2019). There are no major differences between these outcomes and the outcomes in the specialized clinics in Sweden and other countries (Södersten et al., 2017, 2019).

What explains these low remission rates? Consider the most recent RCT in which 15 out of 36 patients (42%) went into remission from bulimia but not from anxiety (Poulsen et al., 2014). On the standard perspective, anxiety causes bulimia (Kaye et al., 2004) and it is unsurprising, therefore, that 5 of the 15 patients (33%) relapsed within 19 months. A new review found no "relevant new RCTs" and concluded that CBT is "an effective approach" (Slade et al., 2018), despite the fact that 22.2% of the patients dropped out, 33% relapsed and 39.3% received additional treatment during follow-up in the trial (Poulsen et al., 2014). Considering that there is no information of long-term outcomes, it should be possible to improve the effectiveness of CBT (Södersten et al., 2017; Slade et al., 2018). A new perspective might offer a start.

THE EVOLUTIONARY PERSPECTIVE

A framework for anorexia nervosa, the prototypical eating disorder from which the other eating disorders emerge, was launched in 1996, with food restriction as the main cause (Bergh and Södersten, 1996). The neuroendocrine changes associated with this brain-based model have been reviewed (Bergh et al., 2002, 2013; Zandian et al., 2007; Södersten et al., 2008, 2016, 2017) and can be briefly updated as follows.

Because starvation has been the main threat in evolution it is fitting to paraphrase Dobzhansky: "Nothing in the biology of anorexia makes sense except in the light of evolution" (Dobzhansky, 1973). And 36 years ago, it was realized that the conspicuous high physical activity of anorexia is a normal, evolutionary conserved response, i.e., foraging for food when food is in short supply (Epling et al., 1983). Later on, the evolutionary perspective was presented twice more (Guisinger, 2003; Södersten et al., 2008).

In fact, anorexia provides an example of the human homeostatic phenotype, as this concept emerged from the clinical observations and hypotheses of Bernard and the subsequent experimental verifications of Cannon (Södersten et al., 2008). This perspective has now been validated for brain function. Thus, the signaling molecules of the hypothalamic arcuate nucleus support the search for food, rather than eating (Ammar et al., 2000; Nergårdh et al., 2007; Chen et al., 2015; Dietrich et al., 2015; Burnett et al., 2016). The agouti-related protein neurons of this nucleus can monitor the availability of food in the environment, changing energy utilization from fat to carbohydrate (Chen et al., 2015; Burke et al., 2017; Cavalcanti-de-Albuquerque et al., 2019). Silencing these neurons eliminates the search for food but leaves chewing and swallowing unaffected (Thomas et al., 2018),

replicating the effect of dopamine receptor blockade or depletion (Berridge et al., 1989; Bednar et al., 1992; Qian et al., 1998).

The search for food and eating behavior, chewing in particular, have dominated the evolution of the behavior and the anatomy of the individual, including the head and the brain (Lieberman, 2011, 2014; Ungar, 2017; Smith, 2018). "You are How you eat," suggests the evolutionary biologist and even that we should "encourage [our children] to chew more gum" (Lieberman, 2011). And since it was first reported that chewing gum is relaxing 80 years ago (Hollingworth, 1939), it is now recognized that chewing gum promotes both physical and mental health (Fukushima-Nakayama et al., 2017). The neural engagement in these beneficial effects of chewing include the serotonin cells in the dorsal raphe nucleus in the brainstem and their projections to the prefrontal and orbitofrontal cortex (Ioakimidis et al., 2011). These serotonin neurons and the hypothalamic agouti-related protein neurons also activate dopamine neurons in the ventral tegmental area in the brainstem (Davis et al., 2011; Browne et al., 2019). Interestingly, activity in these mesolimbic dopamine neurons can functionally rearrange the connections within the prefrontal cortex (Kahnt and Tobler, 2017). Foraging for food has shaped these cortical and subcortical areas into an extended neural network, parts of which are differentially engaged dependent upon environmental conditions (Kolling et al., 2012; Pearson et al., 2014; Carlén, 2017; Korn and Bach, 2018). Dopamine, of course, plays roles in addition to the one(s) discussed here, some of which are important in evolution, including the management of threats (Miller et al., 2019).

It is well known that in evolution "men hunt and women gather" (Fessler, 2002; Gilby et al., 2017), but it is not yet known how these behavioral sex differences are related to the neural network of foraging. Research on the neuroscience of foraging often use economic rewards and choices, food rewards are less common (Kolling et al., 2012; Shenhav et al., 2016). However, it was observed long ago that the emergence of the prefrontal cortex in primate evolution coincided with improvement of the strategies for food foraging (overview in Genovesio et al., 2014). Gonadal hormone sensitive sex differences have since been demonstrated in the anatomy of the prefrontal cortex and these can be related to sexually dimorphic behavior (Clark and Goldman-Rakic, 1989; Evans and Hampson, 2015). On the evolutionary perspective, it is tempting, therefore, to speculate that these findings are related to the marked sex difference in the prevalence of anorexia nervosa.

The neurobehavioral responses to food deprivation and the corresponding genotype are evolutionarily conserved and consistent with the evolutionary perspective of anorexia nervosa (Södersten et al., 2008; Alvergne et al., 2010; Itskov et al., 2014; Gibson et al., 2015; Sato and Kawata, 2018).

The Elusive Clinical Translation of the Neurobiology of Foraging

But rather than controlling behavior, the neural network just outlined is permissive; the cause of changes in eating behavior is outside of the individual (Södersten et al., 2011; Zandian et al., 2015). For example, the behavioral effects of experimental activation of the brainstem to prefrontal cortex part of the network in one environment are the opposite to the behavioral effect of the same experimental maneuver in another environment (Warden et al., 2012; Seo et al., 2019). Similarly, stimulating the brain with neuropeptide tyrosine makes a rat eat more food when food is continuously available but makes the rat forage for food and eat less food when the availability of food is restricted (Ammar et al., 2000; Nergårdh et al., 2007). These results support the proposed causal role of the environment in body weight regulation and suggest that neuropharmacological intervention may remain ineffective.

In normal circumstances, our biological propensity to eat as much as possible is counterbalanced by the need to forage for food (Södersten et al., 2011). But today the effort to find food is minimal and in the absence of internal controls people need external support in order not to lose control over body weight (Södersten et al., 2008).

Eating Behavior in Treatment

In the clinic, we have therefore outsourced the control of eating behavior and body weight to a machine first described in 1996 (Bergh et al., 1996; Södersten and Bergh, 2014). The patients learn to eat assisted by visual feedback from a computer screen as described many times already and recently in an open access video (Esfandiari et al., 2018). But they are also treated with warmth, their physical activity is reduced and they are supported to resume their social activities (Bergh et al., 2002). An RCT demonstrated the treatments effectiveness (Bergh et al., 2002), which was confirmed by a description of the outcomes at 3 months intervals during treatment and 1, 2, 3, 6, 9, 12, 18, 24, 26, 48, and 60 months after remission in 1,428 patients treated in six clinics in four countries (Bergh et al., 2013). The rate of remission was estimated to 75% within on average 1 year

TABLE 1 | Food intake, meal duration, initial rate of eating, change in rate of eating over the course of the meal (b) and hypothetical maximal food intake (a) in 17 women at admission for the treatment of Anorexia Nervosa (Adm AN) and at remission after treatment (Rem AN) and in 17 healthy women (Healthy).

Group	Food intake (g)	Meal duration (min)	Initial rate of eating (g/min)[a]	Change in rate of eating (b)	Hypothetical maximal food intake (a)
Adm AN	100 (72–160)[b]	10.4 (7.7–13.6)	8.4 (6.2–16.3)[b]	–	
Rem AN	307 (266–351)[c]	9.6 (8.0–10.6)[c]	44.3 (34.4–46.9)	0.06 (0–0.22)[d]	586 (377–684)
Healthy	268 (208–389)	10.7 (10.1–12.2)	39.1 (35.8–46.9)	0.12 (0.05–0.23)	374 (333–444)

Values are median (quartile range). See text for model of eating behavior. [a]Derivative at time=1 min of the estimated function; [b]$p < 0.01$ vs. Healthy or Rem AN; [c]$p = 0.06$ vs. Healthy; [d]$p = 0.015$ (Mann-Whitney U-test).

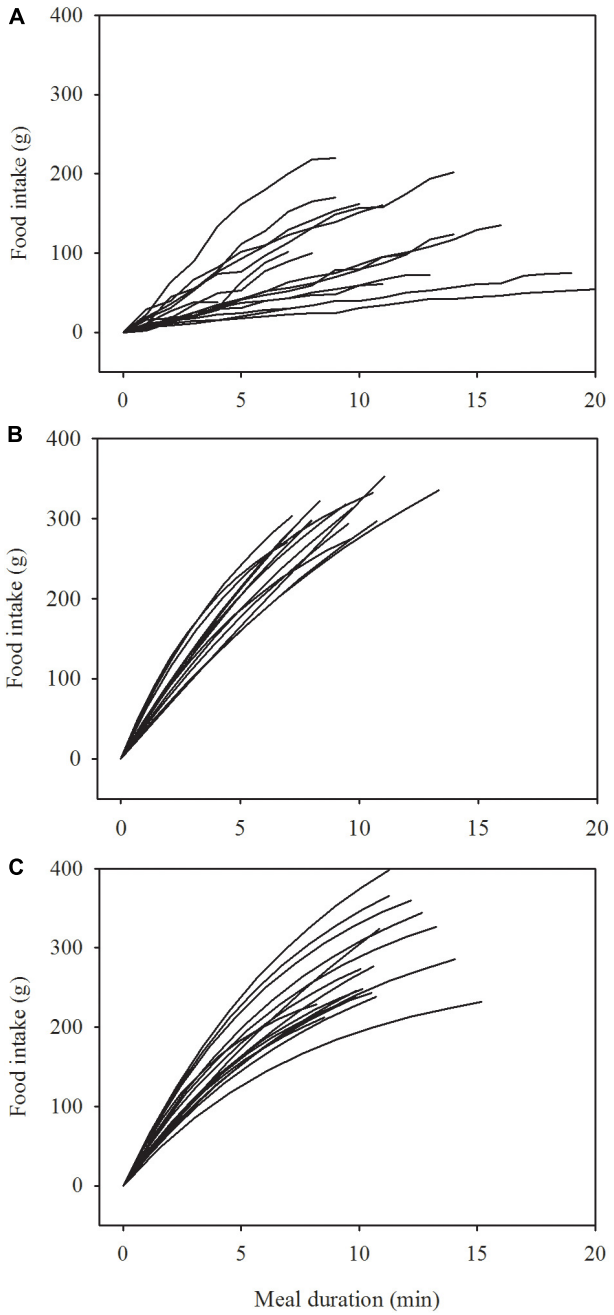

FIGURE 1 | Change in the rate of eating in 17 women at admission **(A)** and at remission **(B)** after treatment of anorexia nervosa and in 17 healthy women **(C)**. Data in **A** are raw data collected at 1 min intervals. Data in **B,C** are modeled by a two-parameter asymptotic exponential curve, see text for details.

of treatment and the rate of relapse was estimated to 10% (Bergh et al., 2013). Psychoactive drugs that had been prescribed prior to admission to treat mental symptoms were withdrawn while the patients remitted from these symptoms by re-learning how to eat (PS and others, manuscript submitted for Gutierrez and Birmingham, 2018).

THE PARADOX OF STANDARD TREATMENT

More patients go into remission in the long-term by re-learning how to eat than if treated with CBT (Södersten et al., 2017, 2019). The difference in outcome is unlikely due to difference in the state of the patients at admission. The published literature suggests the opposite; patients who are treated with standards of care are less serious ill at admission than patients whose eating behavior is treated (Södersten et al., 2016).

Considering the difference in outcomes, it is paradoxical that "the single most effective procedure in CBT" has long been recognized as "*the prescription of a pattern of regular eating*" (Fairburn et al., 1993). But because is unclear how this is achieved we have invited CBT-clinicians to use our method for treating eating behavior (Södersten et al., 2017).

HOW TO EAT

The biological, default pattern of eating behavior, a gradual decrease in the rate of eating over the course of a meal, was first described in experimental animals as: $N = Kt^n$; where N = amount of food eaten at time t and K and n are constants (Skinner, 1930) and then modified as an exponential growth curve: $f = c(1\text{-}e^{-mt})$; where f = amount of food eaten, c and m constants and t = time (Bousfield, 1933). A model of human eating behavior was presented as: $y = kx^2+lx$; where y = amount of food eaten, k = change in the rate of eating over the course of the meal and l = initial rate of eating (Pudel, 1971). This model was subsequently confirmed (Kissileff et al., 1982). The recent suggestion that the model should predict outcomes and disclose mechanisms is based on 40 year old experimental results rather than the recent biology of foraging (Thomas et al., 2017). At present, the model remains descriptive, but as outlined here, it can be used in the treatment of eating behavior in patients with eating disorders.

With $k < 0$ in the model, Westerterp-Plantenga launched the term decelerated eating and with $k \approx 0$, she launched the term linear eating (Westerterp-Plantenga et al., 1990). If rats are deprived of food for 4 days, food intake decreases the linearity of eating increases (Bousfield and Elliott, 1934). Women respond in the same manner after merely skipping dinner (Levitsky and DeRosimo, 2010; Zandian et al., 2011).

Linear eaters eat less food yet feel increasingly full when eating at a reduced rate experimentally and they eat more food yet feel less full when eating at an increased rate experimentally (Zandian et al., 2009a). Thus, dieting, the main cause of anorexia, causes linear eating very rapidly and puts women at risk of losing control over food intake. These undesirable effects can be prevented by practicing eating at a decelerated rate (Zandian et al., 2009b). And when women transit from linear eating to decelerated eating their mental state normalizes (Zandian et al., 2009b), just as 737 patients remitted from their mental symptoms by re-learning how to eat (Bergh et al., 2013).

THE EATING BEHAVIOR OF ANOREXIC PATIENTS TREATED TO REMISSION

The derivative of the old model is a line but growth, including cumulative food intake, tapers off. We therefore re-launch the two-parameter asymptotic exponential curve as a minimally redundant model of eating behavior: $y = a(1\text{-}e^{-bt})$, where y = amount of food eaten, a = hypothetical maximal food intake, b = change in the rate of eating and t = time (Bousfield, 1933).

Using non-linear regression (Bates and Chambers, 1992; R Nonlinear Regression, 2019), we describe the eating behavior of 17 women who were treated to remission from anorexia nervosa by practicing how to eat. Their mean (SD) age was 18.8 (3.7) years, they had been ill for 3.3 (2.2) years and had a Body Mass Index, BMI = 14.9 (1.0) at admission. The women went into remission in 359 (78) days, at a BMI = 19.8 (0.9). For a complete list of remission criteria, see (Bergh et al., 2002). Their eating behavior was compared to that of 17 healthy women, who were 23.6 (2.0) years old and had a BMI = 23.5 (1.5). The choice of 5 years older healthy women for comparison was based on the fact that patients who have been treated to remission are followed up for 5 years before they are considered cured (Bergh et al., 2002).

Table 1 shows that the patients ate only little food, slowly, at admission, but when in remission, they ate somewhat more food than the healthy women and the duration of their meal was a little shorter. While the initial rate of eating among the anorexics in remission and the healthy women was similar, the rate of eating decreased over the course of the meal significantly more among the healthy women than among the women in remission. These differences in eating behavior emerge clearly in **Figure 1**. One of the patients continued eating for 37 min at admission, i.e., beyond the 20 min limit displayed (bottom graph in panel A). Three patients ate in a linear manner at remission and their curves are therefore omitted in panel B.

COMMENTS, PERSONAL INSIGHTS AND OPINIONS

While the anorexic women who practiced eating reached a BMI within the normal range and consumed a normal amount of food, their weight and their eating behavior was not the same as those of the healthy women. Our patients are followed for 5 years after treatment, including eleven appointments (Bergh et al., 2002, 2013) and, at present, we are examining if their physical characteristics and their eating behavior more closely resembles those of healthy women once they have completed the follow-up program. Yet, at the present state of knowledge, it is reasonable to suggest that patients with eating disorders should be offered the chance to practice eating using the device that has now restored the physical and mental health of hundreds of patients (Bergh et al., 2013; Södersten et al., 2017, 2019). Eating behavior thus treated makes it less important, albeit perhaps not unimportant, to treat cognitive processes (Södersten et al., 2017), although evidence that these interventions are redundant was presented 31 years ago (Freeman et al., 1988).

Practicing eating restores the levels of hormones thought to cause weight problems in obesity (Galhardo et al., 2012; Södersten et al., 2015), suggesting eating behavior control of hormonal secretion, i.e., the opposite causal relationship to the conventional homeostatic relationship (Lowell, 2019). The bidirectional relationship among brain and behavior, suggested by Darwin (1872) and confirmed in recent years (Woods, 1991; Ramsay and Woods, 2014), provides support for clinical translation of the present perspective.

In 1996 we suggested that eating disorders are eating disorders, rather than mental disorders, and that the patients therefore should practice eating (Bergh and Södersten, 1996; Bergh et al., 1996). At the time, it was thought that this was misplaced and even dangerous (Crisp, 1995), but today, 23 years later, no-one can treat patients with eating disorders in the Region of Stockholm unless a program for restoring their eating behavior is included in the treatment. Such overly long delays before evidence-based interventions are introduced into clinical practice are common (Kim et al., 2013). Policies to shorten the delay would be useful.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Data on eating behavior is part of treatment and the clinical files are kept in the register of the Mandometer Clinic, approved by the Regional Ethical Review Board of Stockholm. Patients entering treatment are informed verbally and in writing that their data might be used in research and if so, it will be anonymized. They are also informed that they can request that their data is not used and that they can leave the treatment any time without giving a reason. Written consent by the patients is not required for analysis of data collected in registries. The data from the healthy women were re-analyzed from a previous, ethically approved study (Zandian et al., 2009a).

AUTHOR CONTRIBUTIONS

PS launched the idea with all authors. UB undertook the statistical analysis and reviewed these with PS in detail and repeatedly. MZ was keeper of databases and clinical files and quality controller at the clinic. CB was a clinical director and supervised all treatments. All authors contributed intellectually to the content and to completing the final version of the manuscript which has been approved accordingly and wrote and reviewed the manuscript repeatedly.

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Conflict of Interest Statement: Complete openness concerning financial arrangements is intended here. UB and MZ declare that they have no financial interests related to this study. Our research is carried out at the Karolinska Institute, where PS is an emeritus professor. The research is translated clinically by Mando Group AB, a company started by PS and CB, who have 47.5% of the stock each. Professor Michael Leon of the University of California at Irvine has 5%. Mando Group AB contracts with the County Council of Stockholm every fifth year to treat patients with eating disorders. Mando Groups AB signed its first contract in 1997 with the County Council of Stockholm and, since then, its treatment is one of the standards of care treatments offered to the citizens of Stockholm. This arrangement is the same as when the County Council of Stockholm contracts with its own clinics to treat patients with all kinds of disease, including eating disorders. That is to say, the County Council of Stockholm provides eating-disorder services to the citizens of Stockholm both through a clinic of its own and through Mando Group AB. Until recently, there was a third provider of care for patients with eating disorders in Stockholm, which was a private clinic. Mando Group AB is the biggest provider of eating disorders services in Sweden as of 2019. All health care in Sweden is funded through the tax system; private pay is extremely uncommon. It should be added firstly, that Mando Group AB is in compliance with the recommendation of the International Committee of Medical Journal Editors on "Author Responsibilities-Conflicts of Interest" http://www.icmje.org/recommendations/browse/roles-and-responsibilities/author-responsibilities--conflicts-of-interest.html. Secondly, it should also be added that all profit that Mando Group AB has made has been re-invested in research and development and that there have been no dividends to stock owners. All of the above is declared in all manuscript submissions and thus far, journals have judged it necessary to publish only some of the details. It seems, however, that the potential ethical problem when scientists translate their research findings into the clinic in a company is not unlike that which arises when any scientist, in an academic setting is developing a theory and needs further economic funding for her/his work and may receive recognition and financial benefits for the work. The incentive is, in part, economic in this case as well and the ethical "problem" is similar in both cases. However, the more important incentive is the improvement of the treatment of patients with eating disorders. We are researchers working in an academic setting and like many other medical research institutes today, the Karolinska Institute encourages scientists to translate their research into the clinic in companies that aim to generate financial profits to be used for research and development (see https://issuu.com/karolinska_institutet/docs/ki_strategy2030_eng).

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METHODS
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Toward Quantifying the Psychopathology of Eating Disorders From the Autonomic Nervous System Perspective: A Methodological Approach

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The phenomenology of Eating Disorders (ED) relates with altered functioning of the Autonomic Nervous System (ANS). The lack of agreement in what comes to the direction and significance of such alterations is possibly due to the variability in the ED spectrum. As the stress response system is an integral part of the ANS, we propose to investigate ANS tonic variations and phasic activations in response to stressors. We hypothesize that, while using stress as a test probe, characteristic ANS dysregulations in ED may be found when considering several physiological signals measured over time, and weighted by the individual psychological profiles. In this article we describe a novel methodological approach to investigate this hypothesis with the aim of providing further clarification on the ED spectrum conceptualization. The proposed methodology has been designed to be easily integrated in clinical practice and, eventually, in daily life. The population under observation includes both patients in treatment for ED, and matched controls. The study session has the duration of 1 day, including: (1) the administration of a stress task in a controlled environment and (2) naturalistic data collection. The stress task is designed to elicit both mentally and physically driven ANS activation. The naturalistic component intends to illustrate the psychophysiology in everyday life. We use wearable devices to continuously and non-invasively measure bio-signals related to ANS functioning. This information is complemented with psychometric information from validated stress and ED scales and ecological momentary assessments. The protocol has received ethical approval and has been implemented in practice, currently accounting for 37 patients (out of 120) and 16 controls (out of 60). Ongoing work focus on the definition and implementation of a data processing pipeline to quantitatively test our hypothesis, both standard statistical methods and more exploratory machine learning approaches will be considered.

Keywords: eating disorders, autonomic nervous system, psychometrics, ecological momentary assessment, wearables, stress

INTRODUCTION

Anorexia Nervosa (AN) and Bulimia Nervosa (BN) constitute the official ED diagnoses according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)[1] (American Psychiatric Association, 2000). Partial AN/BN syndromes and the prominent Binge Eating Disorder (BED) are included in a residual and highly heterogeneous subset of ED, the Eating Disorders Not Otherwise Specified (EDNOS). ED markedly influence the physical and mental well-being of sufferers. AN is associated with intense fear of fatness, distorted body image, and exaggerated dieting, leading to severe weight loss. BN features recurrent food binges followed by compensatory behaviors (e.g., purges, excessive exercise, or fasting) to avoid weight gain. BED regards frequent episodes of fast intake of exaggerated food portions, accompanied by feelings of lack of control (American Psychiatric Association, 2013a). While not well-documented by epidemiological studies, it is systematically reported that most ED cases fall in the EDNOS group (Machado et al., 2012; Smink et al., 2012; Vo et al., 2017). We know that only a minority of ED cases reaches the health care system (Keski-Rahkonen and Mustelin, 2016), even though most sufferers receive treatment related to unspecific emotional problems (Hudson et al., 2007). ED etiology is multifactorial with more than 70% of patients having psychiatric comorbidities and often switching across diagnosis within the ED spectrum over time (Hudson et al., 2007; Larranaga et al., 2012; Keski-Rahkonen and Mustelin, 2016). Remission occurs after at least 4 years (Keel and Brown, 2010; Kessler et al., 2013). Therefore, in order to comprehensively improve treatment outcome, we may need to better grasp the seemingly dimensional underlying psychopathological mechanisms of these illnesses.

The official guidelines for classification of psychiatric pathologies are described in the DSM (American Psychiatric Association, 2000, 2013b). Disorders are presented as categories and symptoms are evaluated in a syndromic manner. However, while it has been useful as a basis for communication among clinicians and researchers, the DSM validity has long been discussed, and after several updates it is still flawed (Vo et al., 2017). In general, the categorical conceptualization of mental disorders does not rely on a grounded neuro-psychopathological basis, and has been reduced to a matter of opinion or preference (Haslam, 2003; Williamson et al., 2005). Several studies have explored a dimensional perspective, i.e., the idea of a continuous spectrum, to model mental health disorders (Haslam, 2003) and in particular ED (Williamson et al., 2005). Explorations were grounded solely on information from self-reports and psychometric data (no physiological data). According to preliminary results, both categorical and dimensional perspectives may need to be combined in order to describe the mental health disorders spectrum (Williamson et al., 2005).

[1]This classification was recently updated on the DSM-5 (American Psychiatric Association , 2013), though as the methods described in this article were drawn during the transition period, the DSM-IV-TR (American Psychiatric Association, 2000) notation is still adopted.

Arousal and regulatory systems are among the five Research Domain Criteria (RDoC) appointed as potentially valid dimensional constructs underlying psychopathology (Cuthbert, 2014). The arousal system facilitates interactions with the environment in a context-specific manner and has associations to physical fitness (Huang et al., 2013). In psychophysiology, arousal is defined as the response to a perturbation (i.e., a stressor) that may create a homeostatic deregulation of the organism (Koolhaas et al., 2011). While acute arousal can be accommodated by physiological processes, prolonged activation can result in pathological states.

In the human body, specific neuroendocrine paths are triggered causing arousal (Kemeny, 2003; Lo Sauro et al., 2008). The first one involves the ANS (Kemeny, 2003), a part of the Peripheral Nervous System concerning involuntary bodily processes. The ANS encompasses two branches working in opposition to regulate the body equilibrium: the sympathetic branch associated with arousal and the flight-or-fight (FoF) response, and the parasympathetic associated with rest-and-digest states (McCorry, 2007). The FoF is a full body effort to increase blood flow to skeletal muscle, causing considerable effects at a physiological level. It leads to increased heart rate (HR); vasoconstriction in the gut and kidneys to divert blood to the muscles; bronchodilation to facilitate oxygen uptake and carbon dioxide outtake; increase of glucose and fatty acid concentrations in the blood to leverage metabolic needs; generalized sweating for thermoregulation; pupil dilation to allow more light in and adapt vision to focus in the distance. Considering such phenomena, ANS arousal can be described on the basis of pupillary dilation, muscle activity, cardiac activity, respiration rate, blood pressure (BP), skin conductance (SC), and skin temperature (Kemeny, 2003; McCorry, 2007).

Physiological substrates involved in ANS regulation are altered in ED (Westmoreland et al., 2016), for instance decreased HR positively related to lower metabolic rate (Buchhorn, 2016), HRV is inversely related to body weight (Karason et al., 1999; Mazurak et al., 2011), lower skin conductivity relates to dehydration caused by reduced fluid intake or purge, decreased skin temperature is linked to poor circulation induced by reduced cardiac activity. ED onset is often preceded by a specific stressful event (Wheat et al., 2000; Cabras et al., 2008), and the disease maintenance has also been connected to chronical arousal states, linked to specific contexts triggering psychopathological behavior (Wheat et al., 2000).

Literature studies on ANS function in ED report two main study conditions: (1) baseline observations, e.g., at rest or respecting to slow tonic variations; (2) task induced, e.g., in reaction to a stressor. In summary, most studies point out the existence of baseline ANS dysregulations, that are transversal to AN and BN (Mazurak et al., 2011; Buchhorn, 2016; Chudecka and Lubkowska, 2016; Peschel et al., 2016). These dysregulations in AN and fasting BN have been documented as a parasympathetic overactivation with a correspondent sympathetic withdrawal. Task induced responses, mostly show no differences across healthy and ED samples (AN, BN, BED, and EDNOS) (Vocks et al., 2007; Vögele et al., 2009; Hilbert et al., 2011), though, inconsistencies are reported (Koo-Loeb

et al., 2000). Additionally, AN and BN are better documented than BED and EDNOS in what comes to ANS alterations. Cardiovascular parameters (HR and HRV) are the most well-explored descriptors of ANS function in ED, both at baseline and in reaction to stressors. Temperature, BP, and SC are obtained with short measurement protocols, so their use toward pervasive follow-up is not well-explored. Small sample size is indicated in most studies as a limitation, and short cross-sectional measurements also pose limitations toward fully understanding ANS functioning in ED. Overall these studies recommend that considerations should be given to age, body mass index (BMI), comorbidities, associated clinical complications (e.g., starvation, malnourishment, and low body weight), types of eating disordered behaviors (e.g., vomiting), illness duration (chronic or acute), phase of the disease being considered (Mazurak et al., 2011), disorder subtypes (Peschel et al., 2016), and medications.

Our goal is to investigate the tonic and phasic activation of the ANS across the ED spectrum, to discover associations between the psychopathology and arousal states, eventually recurring to advanced data fusion techniques. In this paper, we describe the methodological approach in the basis of our investigation. Answering questions related to whether ANS dysregulations reflect ED psychopathology, are a side effect of the medical complications of the disease (e.g., vomiting and starvation), or both, is out of our scope.

Our work aims at bridging some of the research gaps found in the literature, namely: (1) the full ED spectrum has only punctually been studied using the same psychophysiological protocol (Vocks et al., 2007); (2) physical fitness is usually not assessed in psychophysiological studies and is especially relevant in ED, as it can vary across extremes; (3) bio-signals from several sources have previously been measured in controlled tasks (Vocks et al., 2007; Vögele et al., 2009; Hilbert et al., 2011), though, conclusions were established based on each source separately, without considering data fusion strategies; (4) finally, there has been no report on the study of ED based on bio-signals collected in naturalistic settings. With the general mindset of providing tools to leverage the clinicians work, while testing possibilities that can enhance the follow-up in naturalistic conditions, we employ tools that could be easily integrated as a part of clinical practice. Ecological Momentary Assessment (EMA) to gather information on activity, food consumption, and feelings, if proved useful, can be integrated in a smartphone application. The wearables used for non-invasive autonomic sensing are the most compact and discreet non-invasive sensors in the market, as compared to sensing modalities, such as autonomic Electromyogram (EMG) or central Electroencephalogram (EEG).

We hypothesize that the ED state may be differentiated from a healthy state using a multiparameter approach to data analysis based on psychological and physiological signals. We also intend to test if ED psychopathology can be indexed based on such information. Both hypotheses will be tested on controlled and ambulant data. The following guiding research questions were established to decomposed and test our hypotheses:

- Do ED patients present characteristic autonomic activation during the laboratory stress task, when compared to healthy controls?
- Do autonomic signals during reported high stress moments, in an ambulant condition, reflect the laboratory analysis results (i.e., do both groups still differ according to the previously identified, parameters if any)?
- Can the four most prominent ED classes (i.e., AN, BN, BED, and EDNOS) be identified based on autonomic signals?
- Based on a data driven approach, relying both on psychological descriptors and ANS signals, can we establish a multiparameter model of the ED spectrum (i.e., AN, BN, BED, and EDNOS) that describes psychopathological state?

In the following sections we will present the experimental design (including study population, protocol description, power calculation) (section Experimental Design), discuss the limitations of our approach and suggest possibilities for further development (section Discussion).

EXPERIMENTAL DESIGN

Toward studying the ANS in ED we devised an experiment that would allow us to capture both tonic and phasic ANS activation using the following conditions: (1) at rest in the lab; (2) in response to a stressor in the lab; and (3) in naturalistic conditions, where both rest and stress situations may occur. During the day of the study participants are administered a stress task in a controlled environment, and afterwards they continue freely with their routines for the rest of the day. Measurements of autonomic physiology using wearable sensors are pervasive throughout the day, and EMA on stress, feelings and activities take place every hour.

The detailed methodology is described in the next sections. The protocol was approved by the Medical Ethical Committee of U. Z. Leuven (Belgium), and the study has been running ever since February 2018, with an amendment accepted on January 2019 toward extending the number of centers where subjects are recruited.

Study Sample

The study sample is composed of a group of ED patients (clinical group) and a group of healthy subjects (control group). Detailed admission criteria for each group are displayed in **Table 1**.

The clinical group includes patients (inpatient, day hospital/program, or ambulant regimen) receiving treatment for their ED at a medical unit in Belgium. In the treatment context, all patients, including the ones admitted at the hospital, have free time for rest or social interactions, and can leave the treatment facilities. Mealtimes are fixed in the inpatient and day hospital situations. To pinpoint characteristic features of ED only subjects in treatment are considered, when there is irrefutable need for clinical attention. To gather information on the psychopathology across the ED spectrum, we include patients with strict diagnoses (i.e., AN, BN, BED), but also those falling within EDNOS. These classifications are primarily provided by the clinician. Since we intend to focus on characteristic ED traits

TABLE 1 | Admission criteria: inclusion and exclusion criteria applied for the selection of the participants on the study.

	Study group	Control group
Inclusion criteria	• Be a female; • Having current ED complaints; • Being currently treated for ED; • Have in between 18 and 50 years old; • Have the ability and will to report on feelings several times during the day.	• Be full-time employee in a company, doing office work; • Match in gender, age and BMI one subject in the study sample;
Exclusion criteria	• Suffer from major chronical medical condition requiring continued medication other than that used in the treatment of the ED and its comorbidities; • Suffer from acute medical conditions; • Suffer from dyslexia or dyscalculia; • Be pregnant; • Carry any implanted devices; • Have conditioned mobility; • Be under the effect of medication that impairs the mental state or lack the intellectual capabilities to an extent that prevent the provision of an informed consent to participate.	• Be a night shift worker; • Have history (past or present) of diagnosed mental health conditions or complaints; • Have major stress complaints; • Suffer from major chronic medical condition that requires continued medication;

and its comorbidities, we exclude subjects with coexistent health issues not directly connected to the core features of ED, such as somatic chronical illness with current impact in daily life or need for continued medication, and acute illness. Knowing from epidemiological studies that women are the major group in ED (Larranaga et al., 2012; Smink et al., 2012), and having empirical evidence that men in treatment for ED are relatively sparse, we opted to include only females to establish a grounded statistical analysis. Age was limited to a period that should be stable in terms of major or definitive endocrine changes. We exclude pregnant women related to their special endocrine state. We exclude carriers of implanted devices (e.g., pacemaker) to avoid interference with the wearables. We exclude individuals with dyslexia, dyscalculia, and conditioned mobility, which could interfere with, the execution of the Stroop-Color Word, the arithmetic and the fitness tasks, respectively.

The control group is composed of full-time employees in a 9 to 5 desk job, a condition that will allow us to compare the period of activity and the activity intensity with that of the patients in the hospital. Individuals in this group cannot display major health issues that impact their daily life or that require continued medication, nor should they have history of mental health issues.

Controls are matched to patients according to gender, age and BMI ranges, in accordance to the scheme in **Figure 1**. The BMI intervals proposed by the World Health Organization (WHO) (World Health Organization, 2018) were considered to establish approximate BMI matches, as exact matches would be unpractical for the extremes found in AN and BED that cannot find parallel in the healthy population. For age matching we consider an interval of ± 1 year around the age of the patient, toward maintaining identical demographics in both groups, under the assumption that the difference of 1 year is not biologically relevant in the range considered. With this scheme we intend to maintain comparable anthropometrics across groups.

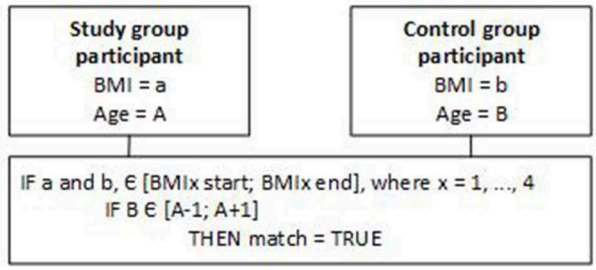
Protocol

The study is conducted over two separate days of contact (see **Table 2**): the recruitment day and the study day. In the first day of contact, the participant indicates the language preference (materials are available in English and Dutch), provides legal consent for data to be acquired and is screened toward verifying the admission criteria. A second day of contact is only scheduled if the participant abides to the admission criteria. The procedures related to the study day take place in the period from 9 a.m. to 5 p.m. The first 1 h 30 min takes place in a controlled environment, during which the study procedures are introduced, devices are setup, the participant is asked to fill in a set of standardized psychometric questionnaires and is administered a multimodal stress task. For the next 6 h 30 min, the participant continues a regular daily routine in ambulant environment. Measurements of autonomic physiology using wearable sensors are pervasive during this day and EMA on stress, feelings and activities takes place at each hour. In the clinical group weight is assessed during this day. In the control group, the weight is evaluated on the first visit to allow the BMI match to be found, and then assumed stable across sessions. The clinician (or the patient) provides a clinical report on the symptomatology being experienced and the type of ED being managed. Both groups abide to similar procedures, described in a standard operating procedure that guides the researchers and clinicians involved in the study.

Sensing Devices

Two wearable sensors are employed in this study: (1) the Chillband (IMEC vzw, Belgium) is a wrist worn sensing device that captures SC, skin temperature and acceleration (**Figure 2A**), at sampling frequencies of 256, 1, and 32 Hz, respectively; (2) the Electrocardiogram (ECG) patch (Biotelemetry, Denmark) is a sensing node attached to an adhesive chest patch, it captures ECG and acceleration (**Figure 2B**) at a sampling frequency of 256 and 32 Hz, respectively. Both wearables were previously employed in

A



B

BMI intervals (kg/m^2)		
Denomination	**Start**	**End**
BMI1	-inf	18.49
BMI2	18.50	24.99
BMI3	25.00	29.99
BMI4	30.00	+inf

FIGURE 1 | BMI and age matching schemes: **(A)** pseudo-code; **(B)** BMI ranges according to WHO.

TABLE 2 | Study overview: recruitment day and study day.

Recruitment day (1 h)	Study day (1 h 30 contact + 6 h 30 free living)
• Informed Consent Form • Verification of admission criteria • Structured diagnostic interview	• 9:00: introduction to study procedures, sensor setup • 9:15: standardized questionnaires, 1st diary (EMA) report • ∼9:45: stress task • ∼10:30: ambulant • 17:00: diary and sensors are collected, participants are debriefed, remuneration is delivered, clinical information is retrieved.

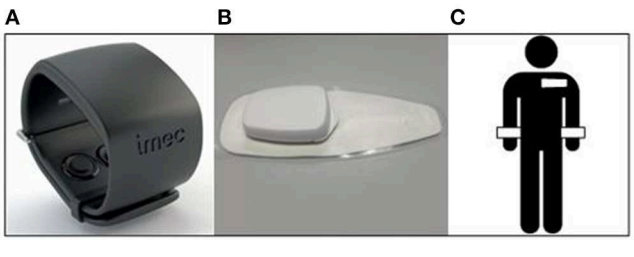


FIGURE 2 | Wearable sensors: **(A)** Chillband, **(B)** ECG patch, and **(C)** sensor positioning.

a large-scale study on daily stress in office workers (Boucsein et al., 2012). These wearables were the most compact and discreet non-invasive sensors in the market at the time of the trial setup. Despite being relevant and frequently used in laboratory settings, EMG and BP are less convenient for measurements in daily life and they were not considered for this study. During the study day participants carry three devices: two Chillbands and one ECG patch, placed according to the depiction in **Figure 2C**. The rational of employing two wrist sensors lays on the fact that wrist SC has been shown to diverge across both sides of the upper body (Picard et al., 2015), and this can provide important information on the arousal states in ambulant conditions.

Clinical Information

In the clinical group, the clinician responsible for the patient fills in a form with clinical information, including the treatment program (i.e., inpatient, day hospital/program, ambulant), days since admission, age, height, weight at start of treatment, weight on the day of the study session, diagnose according to clinical evaluation, symptomatology, and current medication. In the case of ambulant patients, if the clinician cannot be reached, the form is filled in by the researcher based on the patient report.

Standardized Psychometric Tools

Four standardized psychometric tools are employed in this study to model the participants' psychology: the MINI 5.0, a diagnostic interview; the EDI3 (diagnostic list and scale), a self-report to assess ED psychopathology; the DEBQ, a self-report to assess general eating style, and behaviors; and the PSS10, a self-report on stress. These tools have been highly validated and are widely used in trials. **Table 3** describes each of them and their purpose in the context of this study.

Ecological Momentary Assessment

Ecological Momentary Assessment is a psychometric method intended at overcoming the limitations of retrospective self-reports, that rely on recollection of past events and may be biased. This is accomplished by regularly assessing the individuals' state with short questions, providing a close to real time assessment in a naturalist setting (Keel and Brown, 2010). In the past decade, EMA has been increasingly used to study ED, it has been shown useful in enhancing the empirical understanding on the disease and is regarded as powerful instrument to devise personalized and just-in-time interventions (American Psychiatric Association, 2013a).

In this study the EMA consists in a paper diary (cf. **Figure 3**) that is filled in at every hour, from 9 h 30 to 16 h 30, totaling eight reports. As a reminder, an hourly alarm is set on the participant's phone. The diary includes a one-time report about consumption, nutrition and sleeping habit (Buysse et al., 1989), and assessment of events occurred during the past day that may affect the study results (e.g., abnormal sleep duration, alcohol consumption). The first encompasses: activities; food, drink, and medicine consumption; smoke; food purging, overeating, and avoidance contemplation; ratings of stress on a Visual Analog Scales (VAS); and a verbal description of the previous hour. The second includes a momentary evaluation of ratings of stress [VAS and self-assessment manikins (Lang and Bradley, 1994)] and feelings toward food according to three dimensions: physical fullness, mental satisfaction, and intent to eat (Jiménez-Cruz

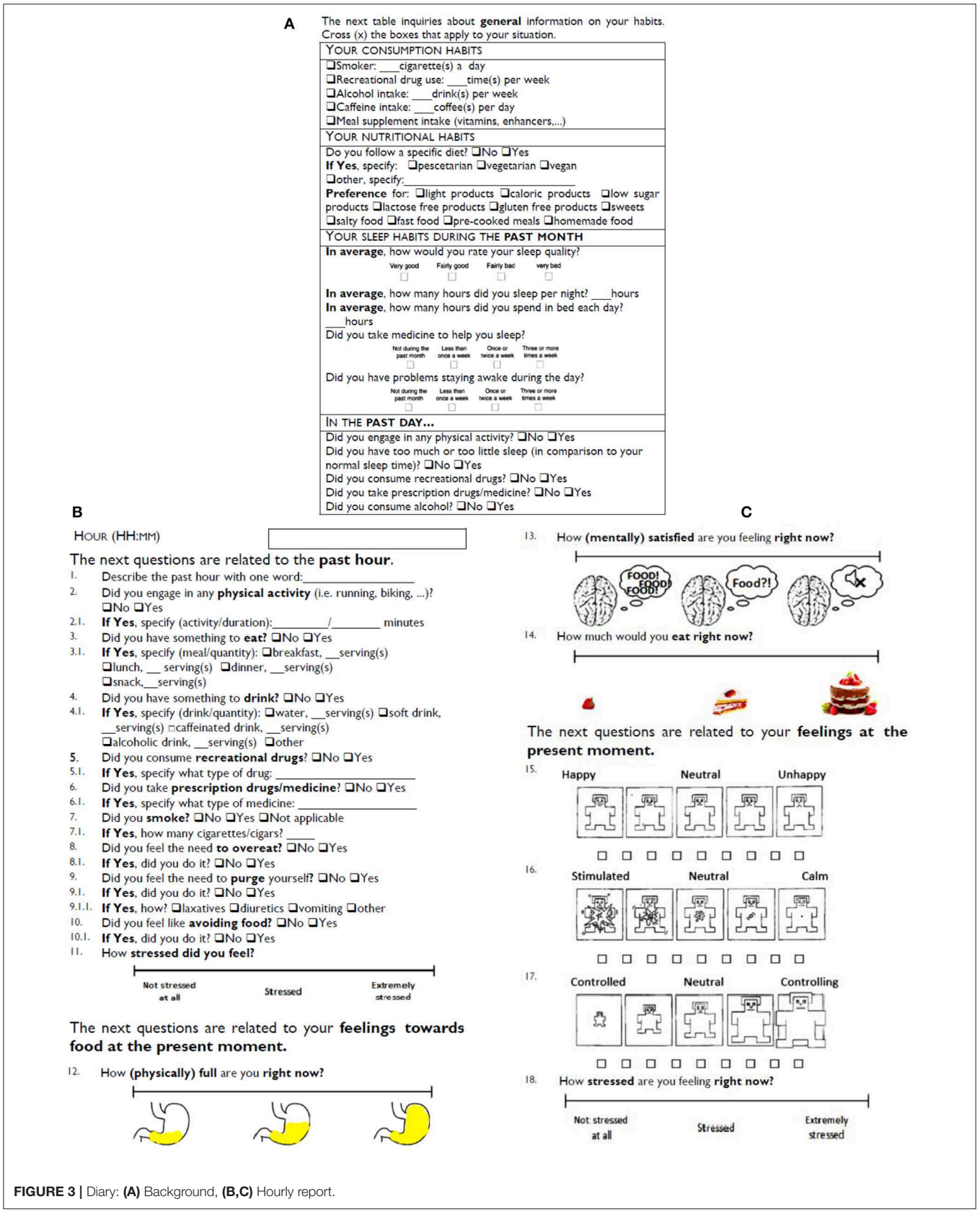


FIGURE 3 | Diary: **(A)** Background, **(B,C)** Hourly report.

TABLE 3 | Description of standardized psychometric tools and their purpose in our study context.

Tool	Description	Purpose
Mini International Neuropsychiatric Interview (MINI)	• Structured diagnostic interview to asses mental health disorders (Sheehan et al., 1998) according to DSM guidelines, with yes/no answers. • Takes 15–60 min to complete, depending on the answers. • Scores high in validation and reliability Lecrubier et al., 1997, and it has been widely used in research.	• MINI-screen version 5.0 is employed to refute the existence of mental health disorders in the control group[a], and to assess the ED typology and psychiatric comorbidities in the clinical group.
Eating Disorders Inventory (EDI3)	• Fifteen minutes self-report focusing on psychological dimensions with clinical relevance for ED. Includes a diagnostic list appendix to assess demographics and anthropometrics. • It is one of the most used tools in ED research Túry et al., 2010 and its psychometric properties are satisfactory Clausen et al., 2011.	• EDI3 and the diagnostic list are delivered to both study groups to quantify the ED psychopathology.
Dutch Eating Behavior Questionnaire (DEBQ)	• Ten minutes self-report. • Includes three scales (emotional, restrained and external eating) relevant to characterize eating behaviors. • Proven successful in assessing eating style traits that are relevant for ED assessment Wardle, 1987.	• DEBQ is employed to gather a general description of eating behaviors, as EDI3 concepts are specific to ED and may be extraneous to non-ED subjects • It is delivered to both study groups, but is specially relevance to describe controls, who may present behavioral nuances not captured in EDI3.
Perceived Stress Scale (PSS10)	• Assesses individual stress levels during the past month. • Five minutes self-report consisting of 10 questions, inquiring how often a certain feeling was experienced on a scale of 0 (never) to 4 (very often). • Considered an effective indicator of the degree in which life events are perceived as stressful Kamarck et al., 1983.	• Used toward quantifying stressful events that occurred prior to the study. • This information is not covered by ED related assessments. It goes along with our view on the connection of stress, ANS dysregulations and ED.

[a] *If a control subject fulfils the symptoms for a MINI diagnosis then she is excluded, even after passing the initial assessment.*

et al., 2006; Forde, 2018). Each hourly report takes less than a minute to complete.

Stress Conditioning

We propose a customized stress task designed to elicit both mental and physical stress-related ANS activations. This multimodal task integrates mental arithmetic stimuli (Stroop Color-Word and calculation test), idiosyncratic stimulus (stress talk), ED specific stimulus (food cues test), physical stimulus (physical fitness task), baseline, and recovery rest phases (**Figure 4**). It takes ∼60 min to complete. The modules and associated timings are indicated in **Table 4** and a detailed description of each module is provided in **Figure 4**.

The task is conducted in a dedicated room and monitored by a trained researcher. The task is displayed on a computer screen, each phase is temporized, and the progression is automatic. The screen is recorded (no audiovisual involving the participant is recorded) to appropriately annotate the sensor data according to the successive activities. Instructions on the activities related to each task module are provided on screen and vocalized by the researcher. The start of the experiment consists of a calibration procedure for subsequent alignment of signals across sensors. It consists of five sets of downward/upward torso movements, conducted while seating, and keeping both hands on top of the sensor on the chest. Relaxing sounds are reproduced during baseline and recovery phases, to calm the participant before and after each stimulus. The researcher keeps a neutral posture, and is allowed only standard interactions during the stimulus, such as *You have to speak faster*; *Please, continue*; *Don't give up*; *You can continue*. Performances are not evaluated during the task. At the beginning and end of the task, participants rate their stress levels and feelings toward food on a short self-report that includes questions 12–18 of the diary (**Figure 3**). The block of activities involving speaking (Counting task, Stroop Color-Word test, Calculation test, Stress talk) and the block of ED relevant stimulus (Food Cues Test) are switched for half the participants, to control for effects on bio-signals related to habituation to the study environment.

Debriefing and Compensation

At the end of the study day the participants must return the wearable devices and the diary. The participant is debriefed and if the diary contains at least eight reports, the participant receives a 10€ voucher for online shopping, as an appreciation for the participation.

Power Analysis

Power analysis estimation was performed to back up the study decisions in what comes to the size of the study sample. Toward this end we considered some simplifications and assumptions:

- We focus only on processing autonomic signals (no psychological variables).
- All autonomic signals will be analyzed toward extracting parameters to describe the ANS function (e.g., mean HR at baseline).
- Each participant will be treated as an observation, represented by a set of parameters (e.g., mean rest HR, mean HR during task, mean temperature from 10 h 30–13 h 30, mean SC at lunch time...).

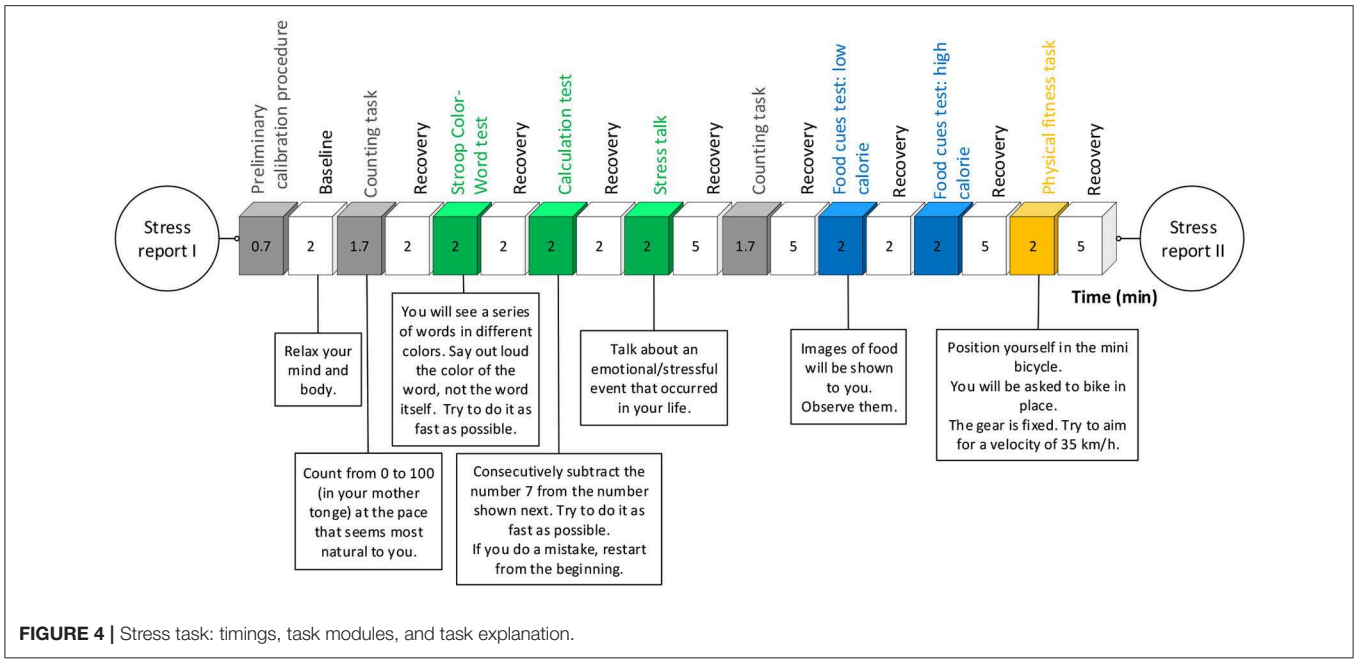


FIGURE 4 | Stress task: timings, task modules, and task explanation.

TABLE 4 | Detailed description of the stress task modules and their purpose in our study.

Task	Description	Purpose
Counting task	• Participant counts from 0 to 100 at a relaxed pace. • This task takes place both at the beginning and at the end of the block involving speaking.	• Control for the effect of speaking in the signals captured by the sensors. • Not supposed to induce stress.
Stroop Color-Word test	• Depicts how humans read faster than they identify and name colors. • Participant is asked to name out loud, as fast as possible and under a limited time, the color of a word presented on the screen, while the word itself designates another color.	• General mental activation. • Social stressor. • Previously, it was effectively used as a laboratory tool for stress induction Blondin and Renaud, 1997.
Calculation Test	• Given a starting number displayed on the screen, the participant is asked to consecutively subtract the number seven from it, as fast as possible, while speaking out loud the result. In case there is a mistake, the participant has to restart the subtraction from the beginning.	• General mental activation. • Social stressor. • Effectively used as a in clinical and healthy samples to induce psychophysiological activation Smets et al., 2016; Huysmans et al., 2018.
Stress Talk	• Participant is invited to speak about one stressful or emotional event that occurred in her life. • Researcher rates how stressful the content of the talk was on a VAS.	• Idiosyncratic mental stimulus. • Effectively used as a in clinical and healthy samples to induce psychophysiological activation Smets et al., 2016; Huysmans et al., 2018.
Food Cues Test	• Visual cues, i.e., pictures, containing food items of low caloric and high caloric foods are consecutively presented on the screen, the participant is asked to observe them.	• ED relevant mental stimulus.
Physical Resilience test	• Consists on cycling in place on a mini-bicycle while trying to achieve and maintain the velocity of 35 km/h.	• Mild physical task is performed to test the physiological response to physical activation.

- We assume a Gaussian distribution for all parameters.
- When comparing clinical and control groups only matched participants will be considered, hence in this situation both groups will have the same number of individuals.
- Gender, BMI, and Age are controlled by design, and activities during the stress task are typified.
- The clinical group has four ED subgroups and the control group has no subgroups.
- Both clinical and control group include a varied set of individuals, we assume these groups to present comparable parameter dispersion. When considering clinical subgroups, the dispersion of parameter values per group should be lower (i.e., individuals are more alike) except for EDNOS, which should have similar dispersion as the overall clinical group.

We drafted four analysis methodologies in accordance to the research questions proposed in the introduction section, and hereafter, we present the respective power calculations:

- Hypothesis 1: *During the stress task, the autonomic parameters from the clinical group differ from those of the control*

group. Toward evaluating the statistical significance of each parameter to differentiate across groups (i.e., unpaired samples) a *t*-test for two samples is considered. The nominal variable is being a patient (true or false), and the measurement variable is one of the autonomic parameters (e.g., mean HR at baseline). We target high magnitude differences across groups [high effect size[2], $d = 0.8$ (Cohen, 1988)], using a strict significance level[3] ($p = 0.01$), with high power[4] ($P = 0.85$). In this situation, using R toolboxes for the calculation (*R.pwr* library), the minimum sample size attained is 43 individuals per group.

- Hypothesis 2: *During an ambulant condition, the autonomic parameters from the clinical group differ from those of the control group*. We use the same formulation as in the previous hypothesis, assuming a *t*-test for two samples. Since data collected in uncontrolled conditions is prone to artifacts, this may affect the signal physiological content and increase the data variance. Therefore, we expect to find less sharp differences, hence we set a lower effect size in this case. We target medium magnitude differences across groups [medium effect size, $d = 0.7$ (Cohen, 1988)], using a strict significance level ($p = 0.01$), with a high power ($P = 0.85$). In this situation, we require at least 55 individuals per group (according to *R.pwr* library).
- Hypothesis 3: *Patient groups (AN, BN, BED, EDNOS) present different autonomic parameters*. We expect to find differences across patient groups based on autonomic signals throughout the full collection. If we assume the symptomatologic classification to have a parallel on physiology, then we expect to be able to find four distinct groups in the data, i.e., AN, BN, BED, and EDNOS. In this context we can use a one-way ANOVA test to verify the statistical significance of differences across groups. Targeting high magnitude differences [high effect size[5], $f = 0.4$ (Cohen, 1988)], using a strict significance level ($p = 0.01$), with a high power ($P = 0.85$), we require at least 29 subjects per group (according to *R.pwr* library).
- As for the fourth research question, related to data driven clustering and classification of ED patients, both unsupervised and supervised machine learning techniques will be used and compared. The diagnostic classes (AN, BN, BED, EDNOS) will be considered as labels for the supervised methods. Power calculation for this analysis was omitted given its exploratory character.

In summary, to attain statistically relevant findings from this study we require at least 55 ED participants and respective controls to test hypothesis 1 and 2, and 116 ED participants to test hypothesis 3. The number of possible participants is highly variable, but we envision that in average two new clinical participants will be available on a weekly basis, and one new healthy match will be found. Considering that the time limit for the study is 60 weeks (15 months) related to project constraints, we will be able to include 120 ED participants and 60 controls. This scenario agrees with the numbers from the power analysis.

Data Management

The data acquired in this study is stored and managed using Research Electronic Data Capture (REDCap) (Harris et al., 2009), a secure web-based tool to support research studies hosted at the University of Leuven. Data input and verification are performed by two different researchers to ensure data integrity. No direct identifiers such as name, phone number, or e-mails are kept in the REDCap database, and the data is pseudo anonymized according to a customized procedure before further processing. Local copies of the data are kept in encrypted hard drives and backed-up once a week.

Data Analysis

A preliminary procedure for data analysis includes the generation of a set of annotations to contextualize the sensor data (**Figure 5**). Namely, the movement pattern resulting from the calibration procedure (**Figure 4**) is detected by visual inspection on the acceleration data of each sensor, and used to align the data across devices (**Figure 5A**). The task phases are identified by comparing individual frames of the screen recording to image templates. The starting point for relevant data is set to the moment at which the calibration procedure takes place (**Figure 5**, bold green line). Sensor data coherent with the stress task are then annotated based on the timings provided by the screen recording.

Quality of the data, in particular for the ambulant recording, is evaluated both via visual inspection and via automatic methods (Jones and Lederman, 2006; Boucsein et al., 2012; Kocielnik et al., 2013; Orphanidou et al., 2014; Smets et al., 2018).

Tonic and phasic components of the ANS activation are accounted *via* calculation of appropriate physiological parameters (e.g., HR and HRV (Task Force of The European Society of Cardiology and The North American, 1996; Acharya et al., 2006; Castaldo et al., 2015), SC level and responses (Boucsein, 2012) and skin temperature mean and variance) from the signals. For a complete list of the parameters that will be used as a starting point to our analysis (cf. section Power analysis) consult Table S5 on the Supplementary Materials of the article by Smets et al. (2018).

The laboratory data will be analyzed separately toward generating a numerical rating (Guo et al., 2018) of the physical fitness of the participant, that will be used as a covariate in the ambulant data analysis. In addition to age, BMI, comorbidities, symptoms, ED subtypes, and medications (highlighted in section Introduction), also time since admission, fitness, handedness, and actimetry (based on the accelerometer) will be controlled for.

DISCUSSION

While ED are mainly studied according to their psychological dimension, we proposed a new approach in which we intend to explore ED relation to ANS dysfunctions. To this end

[2]Effect size phenomena is described by Cohen's $d =$ (mean of clinical sample-mean of control sample)/average standard deviation. Cohen's recommendations for the *t*-test effect size: small effect size, $d = 0.2$; medium effect size, $d = 0.5$; high effect size, $d = 0.8$.

[3]P(Type I error): probability of finding a difference that actually does not exist.

[4]Power = 1–P(Type II error): probability of finding a difference that actually exists.

[5]Cohen's recommendations for the ANOVA effect size: small effect size, $f = 0.1$; medium effect size, $f = 0.25$; high effect size, $f = 0.4$.

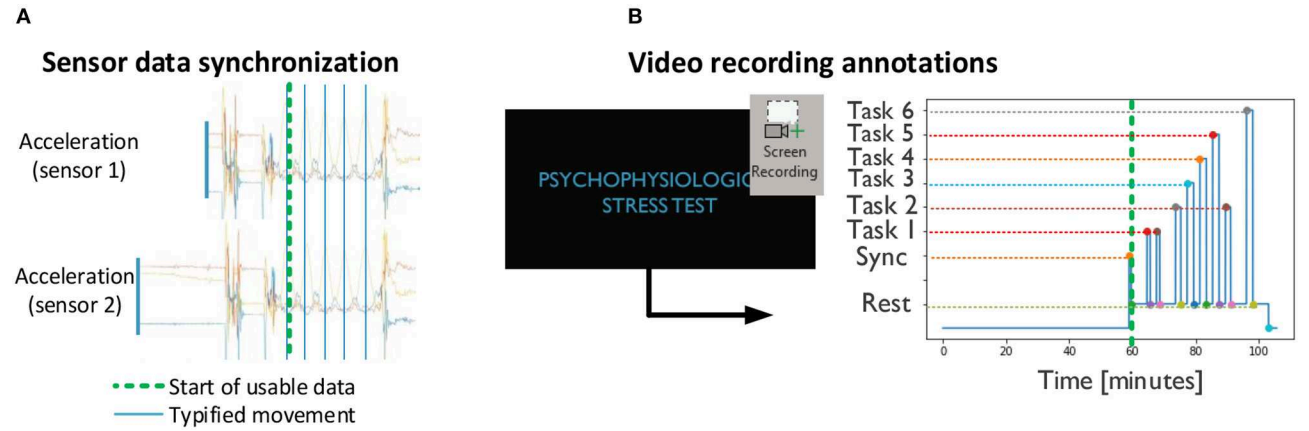


FIGURE 5 | Preliminary procedures: **(A)** synchronization across devices based on acceleration and **(B)** annotation of stress task events based on screen recordings. **(A,B)** are combined according to the sync point (green dashed line).

we designed a comprehensive protocol and successfully implemented it in a study in which we include physiological parameters from ECG, SC, and skin temperature. Participants are studied during the period of 1 day, with the expectation that longitudinal data can provide further insights than cross-sectional data. Recordings take place both in a lab session that calibrate individual physiological responses to different stressors, as well as in ambulatory, allowing participants to do the activities of their choice while the electrophysiological measurements are stored. At the time this article was written, we had collected data on 37 clinical subjects and 16 controls. Ongoing work focus on the implementation of a complete processing pipeline for data analysis and on the quantitative validation of our hypothesis using the collected dataset.

Design Limitations and Notes for Future Enhancement

While the study design presents advantages over a cross-sectional one, because it includes continuous recording over about 8 h, the collection time can be still considered limited. While 1 day of data is enough to test our hypothesis, we cannot exclude punctual factors, not representative of the day to day life of the individual. In future research, conversion of the paper diary into an app and synchronized sensors data streaming directly to a cloud infrastructure, will reduce experimental burdens both for the participants and for the researchers allowing also for longer data collection period and even for easier implementation in clinical practice.

In what comes to physiological sensing, new non-invasive wearables methods have been recently developed that can be used in future trials to complement or substitute the current devices, for example multi-sensor wrist devices[6] for HR, SC, and temperature monitoring, EMG devices[7] to account for muscle tension induced by stress, EEG headsets[8,9] (Emotiv, 2019) already used in arousal studies and attention, eye tracking glasses for monitoring blinking and pupil dilatation[10,11], and hormonal sensing (Stanford EDU, 2018).

With the stress task presented we tried to assess broad set of test situations, nonetheless other variables could have been studied depending on the need to answer specific questions, e.g., emotion perception could be assessed in response to pictures from the International Affective Picture System (Joos et al., 2009; Gorini et al., 2010).

Relevance

In the clinical context the evolution assessment of psychiatric patients relies mainly on self-reports and clinical observation. Information gaps between sparse appointments–when there is no hospital admission—are often bridged based on the patient reports, a recollection exercise that can be affected by momentary altered mental states. In ED, BMI is routinely measured to verify if weight goals are achieved, while cardiovascular assessment and blood analysis may be requested when the physical state of the patient is dubious. In these circumstances, most information gathered about the patient is sparse and subjective, often collected in a non-standardized way. In this backdrop, there is a recognized need for methodological enhancements in the field of mental health management. Nevertheless, in the past decades no major developments have been registered.

[6]IMEC, "Chill+". Available online at: https://www.imec-int.com/en/chill (accessed April 20, 2019).

[7]Plux, "Plux wearables: Muscle BAN." Available online at: https://www.biosignalsplux.com/en/muscleban (accessed May 05, 2019).

[8]IMEC, "EEG headset." Available online at: https://www.imec-int.com/en/eeg. (accessed May 05, 2019).

[9]Emotiv, "EEG headset comparison chart." Available online at: https://www.emotiv.com/comparison/# (accessed May 05, 2019).

[10]IMEC, "Eye tracking with EOG." Available online at: https://www.imec-int.com/nl/eog (accessed May 05, 2019).

[11]Tobii, "Eye tracking for research." Available online at: https://www.tobiipro.com/ (accessed May 05, 2019).

Recently mental health management sprouted attention outside medical and human sciences. This followed the technological developments that led to miniaturization of wireless sensing platforms, enhanced remote communications, increased computational power, and their broad availability to end users. The resulting enhanced possibilities for data collection and processing became an enabling factor for continuous follow-up and multifactorial analysis—seemingly matching the challenges of mental health management. Currently, there is a crescent interest and openness of the psychiatric research community to technology aided mental health management. Nonetheless, the topic is still highly enfolded in speculation and ethical concerns, and solid outcomes need to arise before the clinical community is convinced to adopt new technologies as an integral part of their methods.

If our hypothesis holds, we can further the understanding of psychopathology across ED and help establishing a dimensional biologically oriented nosology. For design reasons our study focuses on the treatment phase, when the patient has already reached the healthcare system. Which is nonetheless, a valid phase to introduce aiding technologies, namely, to help determining the patient psychopathological state, and track it during follow-up, toward modulating the treatment at each step.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Commissie Medische Ethiek, UZ Leuven and in accordance to the Belgian law of 7 of May 2004 with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Commissie Medische Ethiek, UZ Leuven.

AUTHOR CONTRIBUTIONS

NS-C, EV, and GS: conceptualization and investigation. NS-C, EV, CV, and WD: project administration. EV and CV: supervision. NS-C, GS, EV, CV, and WD: writing original draft, review, and editing.

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Induced Pluripotent Stem Cells; New Tools for Investigating Molecular Mechanisms in Anorexia Nervosa

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Anorexia nervosa (AN) is a dramatic psychiatric disorder characterized by dysregulations in food intake and reward processing, involving molecular and cellular changes in several peripheral cell types and central neuronal networks. Genomic and epigenomic analyses have allowed the identification of multiple genetic and epigenetic modifications highlighting the complex pathophysiology of AN. Behavioral and genetic rodent models have been used to recapitulate and investigate, with some limitations, the cellular and molecular changes that potentially underlie eating disorders. In the last 5 years, the use of induced pluripotent stem cells (IPSCs), combined with CRISPR–Cas9 technology, has led to the generation of specific neuronal cell subtypes engineered from human somatic samples, representing a powerful tool to complement observations made in human samples and data collected from animal models. Systems biology using IPSCs has indeed proved to be a valuable approach for the study of metabolic disorders, in addition to neurodevelopmental and psychiatric disorders. The manuscript, while reviewing the main findings related to the genetic, epigenetic, and cellular bases of AN, will present how new studies published, or to be performed, in the field of IPSC-derived cells should improve our current understanding of the pathophysiology of AN and provide potential therapeutic strategies addressing specific endophenotypes.

Keywords: anorexia nervosa, eating disorders, animal models, induced pluripotent stem cells, genetics, epigenetics

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INTRODUCTION: ANOREXIA NERVOSA, A MULTIFACTORIAL DISORDER

Anorexia nervosa (AN), the psychiatric disorder with the highest suicide rate, estimated at 10% per decade, is prevalent in 0.5% of the general population and has a sex ratio of nine females to one male (1). While its genetic heritability has been estimated to be between 50 and 70% (2), environmental factors and epigenetic mechanisms are also thought to be involved in the physiology of AN. Investigations have been performed to analyze the genetic component of AN, either by gene candidate approaches (3, 4) or by genome-wide association studies (5, 6). However, replications of these studies did not necessarily validate previously discovered associations. In fact, the lack of consistency between replication studies has conducted physicians to reconsider the basis of AN, as well as the methods of diagnosis. In fact, whereas the diagnosis is mainly based on patient observation and food intake, most recent studies have demonstrated that AN would be more associated with deregulation of the reward system (7, 8). Genes that have been discovered to be involved in the pathophysiology of AN so far are related to neuroendocrine

regulation, control of digestion, sleep, reward response, and neurotransmission. As the field of epigenetics has attracted a growing interest from the scientific community, especially in psychiatry, where most diseases are not completely determined by genetic causes, several studies have investigated methylation patterns in promoter regions of candidate genes in AN, such as *ANP* (9), *DAT1*, *DRD2* (10), *OXTR* (11), *POMC* (12), and *SNCA* (13), to better understand changes in the dopaminergic systems and hypothalamic pituitary adrenal (HPA) axis. One study investigated the genome-wide methylation profile of peripheral blood samples from individuals diagnosed with AN and evidenced differentially methylated regions in genes involved in development and brain plasticity including the dopaminergic and glutamatergic neurotransmission, and in RNA modifications (14). Although the molecular and cellular mechanisms involved in the pathophysiology of AN require further investigation, genetic and epigenetic studies have highlighted potential genes and mechanisms implicated in the psychiatric and metabolic pathways involved in AN. These developments have in turn promoted the increasing popularity of genetically engineered cellular and animal models for the study of eating disorders. The current review aims to present the added values of these models; and highlight new potential avenues that could be open by the use of human induced pluripotent stem cells (IPSCs).

IMAGING-BASED STUDIES IN COHORTS OF PATIENTS WITH ANOREXIA NERVOSA

Brain imaging techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have been used to better understand the interactions and connectivity between brain regions that may underlie impairments in reward processing, regulation in food intake, and executive functions describing AN (15). Dopaminergic and serotoninergic pathways respectively, involved (i) in reward processing and (ii) in the regulation of aggressive and impulsive behaviors, anxiety, alteration of body perception, and inhibition have been investigated to better decipher the interplay between food restriction and other behavioral traits observed in AN.

An fMRI study showed a group-specific activation of the ventral striatum in AN patients and controls submitted to visual stimuli related to body perception. A higher activation of the ventral striatum was observed in AN patients who received underweight stimuli (16). Interestingly, an increased skin conductance response (SCR) was observed in patients with AN submitted to underweight stimuli, suggesting a higher reward value of starvation in AN. Furthermore, within the AN group, the difference was more pronounced in the Met carriers of the brain-derived neurotrophic factor (BDNF) Val66Met polymorphism (4). The potential implication of genetic vulnerability factors related to the dopaminergic network and impairments in executive function such as inflexibility observed in AN was investigated in patients genotyped for the COMT Val158Met polymorphism, submitted to the Wisconsin Card Sorting Task and to a resting state fMRI. Patients with AN showed a higher level of perseveration, but only underweight patients showed cognitive dysfunction related to the VAL/MET polymorphism. Furthermore, MET/MET homozygous patients within the underweight group showed a higher activation in BA32. These data suggest that starvation affects dopamine degradation, which leads to cognitive impairments in the context of AN (17).

In another study, differences in connectivity were observed in the ventral attention network in patients with AN submitted to a stop–start paradigm, which evaluates motor response inhibition. The responses to external stimuli and connectivity are differentially correlated between controls and patients depending on the 5HTTLPR genotypes. This study suggests that the serotonin pathway may be involved in modulating response to environmental stimuli through changes in the ventral attention network activity in the context of AN (18).

In addition to fMRI-based analysis, studies using PET were performed to further characterize the activation of specific receptors and transporters. D2/D3 receptor binding with [(11)C]raclopride has been quantified in the brains of women who had recovered from AN using PET, with higher binding observed in the ventral striatum and which was correlated with harm avoidance (19). Other studies using PET have shown alterations in 5HT1A, 5HT2A, and 5HTT binding in the frontal, cingulate temporal, and parietal cortices, in the context of AN, which are maintained after recovery (20). These studies show that changes in serotoninergic and dopaminergic neurotransmission observed in AN persist after recovery.

The functional connectivity of brain regions was investigated by fMRI in patients with AN and bulimia who were submitted to a sucrose testing task. In AN, a loss of connectivity from the hypothalamus to the ventral striatum was observed, whereas in control conditions, these connections mediate reward processing related to food intake in response to appetite. In contrast, an increased connectivity between the orbitofrontal cortex and the ventral anterior insula was observed in AN and bulimia. These data suggest that food restriction observed in AN is related to a change in brain region connectivity. The reward related to food intake is supplanted by a decision-based food restriction (21).

ANIMAL MODELS OF EATING DISORDERS AND THEIR LIMITATIONS

Besides the human samples used to investigate the potential genetic and epigenetic components of eating disorders such as AN, animal models have been used to better understand the genes involved in and the mechanism that control the regulation of food intake, reward processing, and eating behaviors that may depend on environmental stress conditions.

The balance between satiety and appetite is in part controlled by neuro-hormones such as neuropeptide Y (NPY), leptin, and ghrelin, which are secreted by peripheral tissues and target brain regions such as the hypothalamus to control food intake. These neuro-hormones also contribute to the modulation of the stress response through changes in cortisol and insulin levels, both of which modify the dopaminergic response in the mesolimbic system, which is involved in reward processing (22). In animal

models, it is mainly the levels of these neuro-hormones and hormones, and their effects on neuronal activity, that have been studied.

Deregulation of the reward system, accompanied by reduced food intake and increased activity, define AN and are largely captured in the activity-based anorexia (ABA) rodent model. This model, achieved by limiting access to food and offering unrestricted access to running wheels, results in weight loss and has revealed deregulations (i) in the opioid and dopaminergic reward circuitry, (ii) in the expression levels of hormones and neuro-hormones, and (iii) in the HPA axis, supporting observations in patients with AN (23). Rodents that have been submitted to ABA present increased endogenous levels of opioids concomitant with decreased food intake (24). Several studies have further investigated the dopaminergic activation in the ABA rodent model. Activation of the mesolimbic reward circuit in ABA rats rescues food intake without changing locomotor activity (25). Treatment of ABA mice with D2/D3 receptor antagonist was shown to decrease symptoms such as hypophagia and weight loss (26). Whereas BDNF, involved in neuronal development and synaptic plasticity, was shown to positively regulate D3 receptor expression in the striatum (27), its expression level assessed in the mesocorticolimbic reward circuit of ABA mice is specifically increased in the ventral tegmental area (28). Interestingly, *BDNF* heterozygous knockout (KO) mice present hyperphagia and obesity (29). These data suggest that control of food intake is interrelated with dopamine activation in the mesolimbic reward circuit. ABA mice also exhibit decreased levels of circulating leptin (positive regulator of food intake) and increased levels of ghrelin (orexigenic molecule), combined with an increased sensitivity to insulin (30). Interestingly, weight loss associated with ABA is reduced by ghrelin administration (31). Furthermore, a decrease of proopiomelanocortin (POMC) has also been observed in rats after 7 days under the ABA condition (32, 33), whereas plasmatic levels of corticosterone were decreased (34). In sum, the ABA model constitutes a stress condition, which leads to an increased secretion of glucocorticoids that modulate dopaminergic transmission in the reward circuit (35).

Models have also been developed to investigate the role of the stress component in eating disorders. Stress has been shown to affect the control of food intake through deregulations of the HPA axis (36). Decreased levels of peripheral ghrelin have been observed following activation of corticotropin releasing factor (37) in mice submitted to a novelty stress. Interestingly, direct administration of corticosterone in the nucleus accumbens, which leads to an increase of dopamine transient (38), is known to activate glucocorticoid receptors (GRs) in dopaminergic neurons from the mesolimbic region (39) and increase the appetite for psychostimulant drugs (40, 41). A study investigating an interaction between genes and the environment was performed on a mouse carrying the human BDNF Val66Met variant. Mice carrying the Met variant and submitted to social isolation at 7 weeks of age are more susceptible to present anorexia-like behavior (42). These data suggest that stressors can both affect the control of food intake and modulate the reward circuit. Another kind of stressor, maternal separation, if combined with a repeated fasting/refeeding cycle, has been shown to generate binge-like eating behaviors in adolescent rats (43). These findings indicate that the developed eating disorder may depend on the applied stressors.

Neuroendocrine changes that may occur in eating disorders have also been investigated in diet or in food restriction models. While hypoinsulinemia combined with an increased sensitivity to dopamine released in the striatum has been observed in animals submitted to chronic food restriction, increased food intake leads to the opposite phenotype (44). Food deprivation also affects the HPA axis by leading to increased levels of adrenocorticotropic hormone and a less efficient negative regulation mediated by corticosterone (45, 46).

Genetic models have also been generated to further understand gene deregulations in the context of eating disorders. The anorexic *anx/anx* mice, which carry a homozygous mutation on chromosome 2 (47), present as normal at birth but rapidly develop hyperactivity and a reduced food intake (48). Importantly, *anx/anx* mice under-eat despite having full access to food, in contrast to other models like ABA rodents. In these mice, peripheral levels of leptin are reduced, and the balance between anorexigenic and orexigenic peptides is disturbed (49–51), with larger populations of cells expressing NPY and agouti gene–related protein (AGRP) in the arcuate nucleus and lower levels of these in the hypothalamic and extra-hypothalamic target areas of the same neurons (52, 53). In humans, variants of AGRP are observed in patients with AN (54), and changes in NPY levels in cerebrospinal fluid are secondary to AN (55). The mice also develop inflammation and degeneration in brain areas near neurons expressing AGRP (56, 57) and have dysfunctional pancreases, resulting in glucose intolerance and high levels of circulating free fatty acids (58)—mirroring findings in people with AN (59). However, one limitation of *anx/anx* mice is that they die approximately 1 month after birth due to mitochondrial dysfunction and neurodegeneration (47).

Similarly, mice with a conditional KO for the tyrosine hydroxylase (*Th*) gene in dopaminergic neurons (60) died 1 month after birth from starvation. These mice were also hypophagic and hypoactive. The lack of food intake in mice with impaired dopamine production suggests interplay between feeding behavior and dopamine-mediated reward processing. While animal models have already provided a substantial amount of information on the mechanisms that regulate food intake and reward processing, it remains very difficult to determine how these two functions, seemingly intersecting each other in the context of eating disorders, are connected in humans. Another limitation remains in matching biological and behavioral observations in these models with the clinical criteria used for diagnosing people with such disorders. Finally, eating disorders such as AN are recognized to be multifactorial diseases, arising from a variety of genetic and epigenetic factors that cannot be fully recapitulated in animal models, where the roles of different genetic and environmental factors can only be studied in relative isolation.

Data collected from genetic, epigenetic, and imaging-based studies on cohorts of patients with AN, and on rodent models, provide information and potential mechanistic insights that can

be further investigated and complemented by research performed on IPSCs.

IPSC-DERIVED CELLS AND EATING DISORDERS

Whereas, most of the studies performed in the field of AN have primarily used DNA to analyze either potential genetic vulnerability or methylation profiles, somatic cells can also be used to further investigate changes observed in any cell type of interest. In fact, somatic cells such as fibroblasts or peripheral blood monocyte cells can be reprogrammed into IPSCs by overexpressing four transcription factors, *KLF4*, *C-MYC*, *OCT4*, and *SOX2* (61). The IPSCs can be induced to neurons or other cell types of the central nervous system (CNS) (62) or other organs. IPSC-derived cells have notably been used to investigate neurodevelopmental disorders (63, 64) for which post-mortem brain tissue is not necessarily accessible.

Hypocretin (HCRT) or orexin is a neuropeptide expressed in the hypothalamus that positively regulates food intake (65). A recent study has been able to generate orexin positive neurons derived from human IPSCs. The authors demonstrated that, in the IPSC-derived neurons, prolonged exposure to glucose induces a silencing of the *HCRT* gene mediated by histone modifications, specifically H3/H4 hypoacetylation. Interestingly, cell treatment with N-acetyl-d-mannosamine, a derivative metabolite produced from glucose, reactivates *HCRT* expression in IPSC-derived neurons (66).

IPSCs have recently been used for the first time to investigate changes in gene expression profiles that may occur in the context of AN (67). The authors performed an RNA sequencing analysis on 4-week differentiated IPSC-derived neurons from controls and AN patients. The IPSC-derived cells obtained were mostly glutamatergic neurons. The authors found that, after correction for multiple testing, in a list of 110 differentially expressed genes (DEGs), some of them encode proteins that are likely to interact together. A functional annotation of the DEG showed an enrichment of genes involved in the tachykinin receptor pathway and in the response to estrogen. As the tachykinin receptor is expressed in dopaminergic structures such as the striatum (68), the change in expression of *TACR1* is likely related to an alteration in reward processing, which is dysregulated in AN. Furthermore, several studies have demonstrated that the tachykinin receptor is likely to mediate a reward response to opioids (69) and alcohol intake (70, 71), and its mediated pathway has being involved in the addiction process (72). Data collected by Negraes et al. provide supplementary evidence that neuroendocrine responses and dopaminergic neurotransmission related to reward processing are altered in AN. Whereas, Negraes et al. studied IPSC-derived glutamatergic neurons from patients, results collected from brain imaging studies on patients with AN and from rodent models have shown changes in serotoninergic and dopaminergic pathways and neurons in the context of AN. These two neuronal cell subtypes, derived from human IPSCs, have to be further investigated in the context of AN (beginning with their transcriptome and methylome), as procedures to generate them are now available (73, 74). Many drugs targeting serotoninergic and dopaminergic neurotransmission have been used to improve behavioral features related to AN (75). Such drugs should be tested on IPSC-derived cells from patients (i) to determine the functional effect of the drug on a cellular and molecular level and (ii) to infer the patient response. On the functional side, as the ghrelin receptor has been shown to form a heterodimer with dopaminergic receptors to mediate the anorexigenic effect of ghrelin (76), the effect of the neurohormones on (i) neuronal activity of the dopaminergic network and (ii) downstream signaling pathways could be investigated using IPSC-derived dopaminergic cells.

Patient IPSCs make it possible to generate and analyze specific cell subtypes that contain most or all of the genetic and epigenetic information potentially relevant to AN, whereas animal models cannot capture the multigenic risk factors mediated by the patient's genetic background.

INVESTIGATING EPIGENETIC REGULATION IN IPSC-DERIVED AND INDUCED NEURONAL CELLS

As epigenetic regulation seems to play a crucial role in AN, being able to generate IPSC-derived neuronal cells from patient samples would permit the analysis of cell type–specific differential methylation related to this eating disorder. A database was recently created containing the complete DNA methylation profiles of blood and brain samples of 16 control individuals (77). Such databases could be used to compare methylation profiles between patient samples and IPSC-derived cells. Regarding the analyses of DNA methylation in IPSC-derived cells, several studies investigating genetically defined neurodevelopmental disorders have highlighted relevant differentially methylated regions that can be compared with genome-wide expression profiles (78–80) and potential disease phenotypes. However, it is important to note that during the reprogramming of somatic cells to IPSCs, the original methylation pattern can be partially lost in specific DNA regions (81–83). Although IPSC-derived cells represent a very powerful tool for the investigation of genetically defined eating disorders, some limitations may be encountered for eating disorders whose genetic component has not been well-established. In order to minimize changes in the methylation patterns during cellular reprogramming, procedures to directly convert blood cells to neurons are currently being improved (84). Such procedures constitute a new strategy to investigate *in vitro* neuronal models of large cohorts. Furthermore, changes in global DNA methylation profiles have been analyzed during the direct reprogramming of fibroblasts to neurons (85) in order to identify modifications of methylation patterns that are specific to those procedures. Relevantly, several studies have highlighted changes in DNA methylation profiles from AN patients' blood cells (86). While the CRISPR–Cas9 system has primarily been used in IPSCs to edit the genome and create or repair mutations within an isogenic background, the system, when coupled to the DMNT3A or TET1 enzymes, is capable of methylating or demethylating specific sequences. The procedure, initially tested

in transformed cells lines (87, 88), has been adapted to IPSCs (89, 90). As an example, targeted methylation of Exon 1 of the *SNCA* gene [whose promoter was found to be hypermethylated in AN (13)] on IPSC-derived dopaminergic neurons led to decreased expression of synuclein alpha (SNCA) (91). IPSC-derived and induced neuronal cells from patients, combined with CRISPR systems, could allow for (i) the identification of specific epigenetic signatures related to dysregulations and behaviors observed in AN, (ii) the investigation of specific treatment responses, as well as (iii) the observation of cellular and molecular phenotypes after restoration of the DNA methylation profile.

CONCLUSION

Eating disorders, including AN, are complex and multifactorial diseases. Investigations performed on cohorts of patients suffering from eating disorders have shown potential genetic associations and epigenetic changes related to the control of food intake and reward processing. Furthermore, animal models have played a key role in beginning to decipher the role of hormones and neuro-hormones in these two affected mechanisms. However, these approaches do not sufficiently permit the investigation of the interplay between regulation of food intake and reward processing and do not recapitulate the complex behavioral phenotypes observed in patients with AN, including life events and the chronicity of this disorder. With this in mind, while methylation changes during the reprogramming steps need to be carefully examined, patient IPSC-derived cells, such as serotoninergic and dopaminergic neurons, combined with CRISPR editing tools, constitute potentially powerful models to further analyze the cellular and molecular mechanisms, gene expression changes, and epigenetic modifications that underlie eating disorders.

AUTHOR CONTRIBUTIONS

GM, NR, and PG designed the manuscript. GM, NR, and ID wrote the manuscript. ID, NR, and PG edited the manuscript.

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REVIEW
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The Ghrelin-AgRP Neuron Nexus in Anorexia Nervosa: Implications for Metabolic and Behavioral Adaptations

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Anorexia Nervosa (AN) is viewed as primarily a psychiatric disorder owing to the considerable behavioral and genetic overlap with mood disorders and other psychiatric traits. However, the recent reconceptualization of AN as one of both psychiatric and metabolic etiology suggests that metabolic circuits conveying hunger, or sensitive to signals of hunger, may be a critical nexus linking metabolic dysfunction to mood disturbances. Within the brain, hunger is primarily percieved by Agouti-related (AgRP) neurons and hunger increases plasma concentrations of the hormone ghrelin, which targets ghrelin receptors on AgRP neurons to facilitate metabolic adaptations to low energy availability. However, beyond the fundamental role in maintaining hunger signaling, AgRP neurons regulate a diverse range of behaviors such as motivation, locomotor activity, negative reinforcement, anxiety, and obsession and a key factor involved in the manifestation of these behavioral changes in response to activation is the presence or absence of food availability. These changes can be considered adaptive in that they promote affective food-seeking strategies in environments with limited food availability. However, it also suggests that these neurons, so well-studied for their metabolic control, shape mood-related behaviors in a context-dependent manner and dysfunctional control leads not only to metabolic problems but also potentially mood-related problems. The purpose of this review is to underline the potential role of AgRP neurons and ghrelin signaling in both the metabolic and behavioral changes observed in anorexia nervosa. We aim to highlight the most recent studies on AgRP neurons and ghrelin signaling and integrate their metabolic and behavioral roles in normal function and highlight how dysfunction may contribute to the development of AN.

Keywords: behavior, anorexia, hunger, appetite, AgRP, GHSR

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NEUROENDOCRINE CONTROL OF ENERGY HOMEOSTASIS

Energy homeostasis is the balance between energy intake, including the total amount and density, and energy expenditure, including basal metabolic rate, diet-induced, and activity-induced thermogenesis. The maintenance of energy homeostasis is an integral process required for the ongoing sustainability and survival of a species, especially since starvation leads to death and metabolic imbalance affects reproductive fertility. The hypothalamus is a key structure involved in the maintenance of energy homeostasis and is composed of different nuclei that contain a

variety of neuronal populations. These nuclei are involved in vital functions such as stress, thermogenesis, reproduction, growth, metabolism, and food intake (1). Key nuclei responsible for energy homeostasis include the arcuate nucleus (ARC), the ventromedial hypothalamic nucleus, the paraventricular hypothalamic nucleus, the lateral hypothalamus and the dorsomedial hypothalamic nucleus (2, 3).

The ARC was first implicated in the control of food intake and glycemia using neurotoxic drug injections (4, 5). Indeed, it was later discovered that these treatments led to the destruction of specific neuronal populations including proopiomelanocortin (POMC)-expressing neurons and agouti-related peptide (AgRP)-expressing neurons. AgRP neurons, the focus of this review, are critical for survival since adult-ablation of these neurons leads to anorexia, rapid weight loss, and death by starvation (6). Further, beyond the fundamental role in maintaining hunger signaling, AgRP neurons regulate a diverse range of behaviors such as motivation, locomotor activity, negative reinforcement, anxiety, and obsession (7–10), and a key factor involved in the manifestation of these behavioral changes in response to activation is the presence or absence of food availability. For example, AgRP activation in presence of food drives food intake, whereas when food is not available it drives other motivated goal-directed behaviors and reduces anxiety-like behaviors (7, 8, 11, 12). These changes can be considered adaptive in that they promote affective food-seeking strategies in environments with limited food availability. However, it also suggests that these neurons, so well-studied for their metabolic control, shape mood-related behaviors in a context-dependent manner and dysfunctional control leads not only to metabolic problems but also potentially mood-related problems.

AgRP neurons are located at the base of the third ventricle near the median eminence and can rapidly sense changes in metabolic state through neuroendocrine feedback mechanisms involving various hormones and nutrients. During hunger or energy deficiency, where the body expends more energy than it receives, elevated plasma ghrelin provides critical feedback information to the brain, signaling negative energy balance (13). Indeed, AgRP neurons are a key target of plasma ghrelin, with >80% of AgRP (coexpressing Neuropeptide Y [NPY]) neurons also expressing the ghrelin receptor (GHSR; growth hormone secretagogue receptor) (14). Moreover, a number of functions ascribed to ghrelin can be attenuated or blocked when manipulating GHSRs in the ARC or after deleting GHSRs from AgRP neurons (15, 16). Thus, in a physiological setting many of the behavioral adaptations caused by AgRP activation maybe be related to ghrelin signaling.

A primary and critical role of ghrelin is to inform the brain of low energy availability. Although GHSRs are found in a number of different brain regions, AgRP neurons remain a primary target to convey this metabolic information via a variety of specific projections (17). Ghrelin-AgRP feedback is specifically designed to prevent excessive and pathological weight loss. This system, however, is not fail-safe, with AN a prominent example whereby patients present with a severe energy deficit and dangerously low body weight.

AN belongs to a family of eating disorders including bulimia nervosa and binge-eating disorder. The pathogenesis of AN involves a number of genetic, neurobiological, psychological, socio-cultural, and developmental factors (18) with accumulating evidence suggesting an important role for metabolic dysfunction (19, 20). Further support for the metabolic origins of AN comes from a recent genome-wide association study that revealed significant genetic correlations with metabolic traits including insulin resistance and glucose metabolism (21). AN patients present various hormonal and neurobiological alterations associated with negative energy balance, leading to the dysregulation of homeostatic systems (22, 23), which is frequently associated with other psychiatric disorders (24–26). Given that AgRP-ghrelin signaling influences both metabolic and behavioral consequences, particularly in the absence of food availability, it is intriguing to speculate that abnormal function of this system may contribute to both the metabolic and behavioral consequences of AN. The purpose of this review is to underline the potential role of AgRP neurons and ghrelin signaling in both the metabolic and behavioral changes observed in AN. We aim to highlight the most recent studies on AgRP neurons and ghrelin signaling and discuss their metabolic and behavioral roles in normal function and discuss how dysfunction may contribute to the development of AN.

AN: PREVALENCE AND PERSISTENCE

The first description of behaviors linked to AN date back the Middle Ages with the case of St Catherine of Siena (27), although it was Sir William Gull who first coined the term AN in 1874 to define a number of his patients (28). Diagnostic criteria for AN comprise persistent restriction of food intake leading to significantly low body weight in the context of what is minimally expected for the height, age and developmental stage of the individual, in addition to a fear of weight gain and becoming fat, and a disturbance of the self-body perception with dysmorphobia. Different studies report the incidence of eating disorders including AN among the Australian or European populations, as <1–5% of the population, and predominantly in females (29–31). AN has long-term and long-lasting effects, as evidenced by a large cohort study following inpatients over 25 years that showed remission in only 30% of patients, with close to 46% in either partial remission or with a crossover diagnosis of eating disorder not otherwise specified (EDNOS) and 16% of patients retaining their AN diagnosis (32).

ENDOCRINE CONSEQUENCES OF AN

Many of the endocrine alterations observed in AN patients are found in all animals in response to prolonged fasting or food restriction in order to meet and maintain energy demands (33, 34). Different phases are classically described in mammals, including humans (33, 35); following a hypoglycaemic period the secretion of glucagon, epinephrine or glucocorticoids, the main counter-regulatory hormones, lead to a glucose overcompensation from glycogenolysis and then

gluconeogenesis mainly from the liver and kidney. Fasting is accompanied by other hormonal alterations including a decrease of plasma leptin and insulin concentrations, in parallel with the increase of plasma ghrelin concentrations. If the fasting is prolonged the organism starts to use stored lipids, causing a marked increase of glycerol and free fatty acids in the plasma, both of which are used by the liver to produce glucose and ketone bodies, respectively (35). Lipolysis, gluconeogenesis and synthesis of ketone bodies caused by severe restriction are all associated with a reduction of energy expenditure, as a means to protect energy stores (36–38). Finally, when lipid stores are completely depleted, an organism enters a proteolytic phase in which proteins from the muscle provide carbon precursors used in the different steps of gluconeogenesis (35). This depletion of energy stores is associated with a reduction of both lean and fat mass and in the most severe situations induces muscle wasting as well as decreased body temperature (39, 40). AN patients exhibit most, if not all, of these physiological consequences of severe calorie restriction, however, it is interesting to note that the BMI used to reflect the severity of the pathology is regularly lower in AN patients compared to starvation and/or food restriction studies in healthy volunteers. For example, it is not rare to observe a BMI lower than 15 $kg.m^{-2}$ in AN patients at admission whereas subjects from the seminal Minnesota semi-starvation study presented as 16.4 $kg.m^{-2}$, on average, after 24 weeks of food restriction (20, 41, 42). This is likely due to the paradoxical increase in energy expenditure that manifests in the majority of AN patients (43, 44). Interestingly, no relationship has been found between the severity of the disease and mood disorder outcomes, although lower bone mass density was observed in more severe cases (41, 42, 45).

Among all the hormones affected in AN patients, changes in leptin and ghrelin may be best used to aid in diagnosis (18, 24, 46, 47). Some euglycemic hyperinsulinemic clamp studies in AN patients have shown significantly lower total ghrelin, suggesting an increase of satiety sensation (48). Other studies have suggested that despite the high levels of plasma ghrelin in AN patients, ghrelin resistance could explain the ability to engage in persistant food restriction (49–52). Significant elevations in plasma AgRP levels have been demonstrated in AN patients compared to controls (53) and subtle impairments in cognitive flexibility associated with acute AN were negatively correlated with plasma AgRP levels (53). Moreover, several genetic, and genome-wide association studies have shown associations between the occurrence of AN and ghrelin-related hormones and peptides including preproghrelin, ghrelin O-acyltransferase (GOAT), the enzyme required for acylation, and AgRP (54–57). Genetic evidence from patients supports a role of AgRP in AN, indicating that allelic variations in the AgRP gene are associated with susceptibility to AN, with one polymorphism conveying a relative risk of 2.63 for carriers to develop the condition (58). Single nucleotide polymorphisms in the melanocortin-3 receptor (MC3R) were proposed to underlie this association, however, direct sequencing of four single nucleotide polymorphisms in the MC3R did not demonstrate significant associations with AN (59).

BEHAVIORAL CHANGES IN AN

AN is often associated with comorbid diagnoses, particularly anxiety and depression (60, 61). Other psychiatric tendencies such as obsessive-compulsive behavior and harm avoidance have also been observed in many patients (60, 61). Besides restrictive feeding behavior, up to 80% of AN patients engage in excessive physical activity in order to reduce their body weight, a behavior that is often considered compulsive (62). In contrast, non-AN subjects that participated in the Minnesota semi-starvation study reported lethargy and a reduction of self-initiated spontaneous activity.

Although the mechanisms need to be clarified, these results suggest homeostatic hunger signals, such as AgRP neuronal activity and plasma ghrelin, may manifest different goal-directed behavioral outcomes in AN patients compared to healthy controls. Both AgRP neuron activity and ghrelin signaling increase motivation, which is usually directed toward a food goal. However, when food is no longer a relevant goal, a shift in goal-directed behavior to locomotor activity may reflect a strategy to channel motivation derived from homeostatic signaling toward non-food related outcomes. In support of this, both AgRP neuron activity and ghrelin signaling increase locomotor activity in rodents when food is unavailable (63–66) and blocking ghrelin/AgRP actions decreases physical activity and/or food anticipatory behavior compared to control animals (67, 68). Moreover, in time-schedule feeding studies, ghrelin is required to promote food anticipatory activity (69, 70) and plasma ghrelin concentrations are positively correlated with food anticipatory activity. Central ghrelin injection also increased anticipation of palatable food (71).

It is regularly reported that stressful life events (e.g., separations, violence, aggression) precede the development of eating disorders (52). Many studies show that perinatal or juvenile stress can predispose individuals to the development of metabolic phenotypes in humans and in rodents (72, 73) and contribute to psychiatric phenotypes (74, 75). These studies highlight that perinatal and/or juvenile stressors can manifest in adulthood as both metabolic and psychiatric problems, reinforcing the important link between metabolic and mood related circuits in the brain. Thus, we put forward the novel hypothesis that early-life stress might impact common neural circuits regulating energy homeostasis and emotional mood responses, which could predispose individuals to both metabolic and psychiatric problems in later life.

THE GHRELIN-AgRP NEURON AXIS IN ANIMAL MODELS OF AN

The homozygous anx/anx mouse model develops the primary symptom of AN, starvation and subsequent emaciation, however dies prematurely around 3 weeks of age, when they weigh around half as much as their wildtype siblings and display a range of hypothalamic neuropeptidergic and molecular aberrances (76), including an increased number of AgRP/NPY immunopositive cell bodies in ARC (77). However, the neuronal circuits

responsible for energy homeostasis are not fully developed during the short lifespan of this model, making it difficult to extrapolate these findings to the neuroendocrine dysfunction observed in AN patients. Although ghrelin resistance is known to occur in obese animal models (78–81), to our knowledge, no study has directly implicated altered plasma ghrelin levels in the anx/anx phenotype or in other animal models of AN. In the activity-based anorexia (ABA) rat model, which relies on allowing animals unhindered access to running wheels in combination with time-limited access to food (82), central infusion of the inverse agonist AgRP (83–132) increased both cumulative food intake and basal body temperature during exposure to ABA conditions, but did not significantly impact body weight loss (83).

In support of the hypothesis that early-life stress might contribute to the development of AN, it has been shown that early-life stress in a mouse model impacts on both leptin and ghrelin secretion and AgRP fiber density, with changes in plasma ghrelin seen only in females (84). Importantly, both ghrelin and leptin play a critical role in the development of hypothalamic circuits regulating feeding and diet-induced obesity impairs hypothalamic NPY and AgRP signaling, as well as POMC fiber pathways (84, 85). Thus, early-life stress can impact on neural circuits controlling energy homeostasis and can predispose individuals to metabolic disease (diet-induced obesity) in adulthood (86–88). Whether or not similar early-life stress events predispose to AN in animal models via homeostatic circuit modification has not been addressed but should be considered in the future.

THE ROLE OF GHRELIN AND AgRP NEURONS IN METABOLISM

AgRP neurons are essential hunger-sensing neurons, as shown by the seminal studies of Luquet et al. (6). In this study, the authors used mice expressing the human diphtheria toxin receptor in AgRP neurons ($AgRP^{DTR}$ mice) allowing the destruction of these neurons after diphtheria toxin treatment. Diphtheria toxin in adult mice caused a rapid and substantial decrease in food intake and body weight, results that have been subsequently confirmed using similar techniques (89, 90). Importantly, neonatal ablation of AgRP neurons did not lead to a pronounced phenotype (6). These results highlight not only the importance of compensatory mechanisms in the neurodevelopmental process of hypothalamic feeding circuits but also the indispensable role of the AgRP neurons in sensing hunger and feeding behavior. As a key hunger signal, ghrelin targets AgRP neurons to increase food intake and although it has been shown that ghrelin requires AgRP neurons to increase food intake, a number of studies demonstrate that other ghrelin sensitive regions, including the hippocampus and brainstem, are also involved in the control of food intake (91–95).

Chemogenetic and optogenetic techniques developed more recently have allowed researchers to comprehensively define this role of hunger-sensing AgRP neurons (8, 9, 96–99). By using DREADD hM3Dq expression in AgRP neurons of NPY and GABA receptor double knockout mice, Krashes et al. (98) showed that both NPY and GABA are necessary for a rapid increase of food intake, whereas stimulating AgRP neurons in the absence of NPY and GABA had a delayed effect on food intake indicating AgRP peptide produces a slower feeding effect than NPY or GABA (98). Besides stimulating food intake, activation of AgRP neurons increases fat mass and reduces energy expenditure, respiratory exchange ratio and body temperature, all of which contribute to the conservation of energy (63, 100, 101).

Rodents, like humans, adopt similar strategies to cope with acute or chronic energy deficit in order to maintain vital signs in homeostatic range and organ functions (102, 103). At the level of AgRP neurons, food deprivation leads to changes in gene expression in pathways involved in hormone signaling, including leptin, insulin and ghrelin that leads to modulation of AgRP, NPY and GABA expression (104). Ghrelin acts on central and peripheral targets via the expression of GHSR1a and, as well as increasing food intake, ghrelin reduces energy expenditure and fat usage, increases glycogenolysis and glycemia (47). Collectively, ghrelin is a metabolic signal that informs the brain of low energy availability, allowing for metabolic adaptations to conserve energy. Ghrelin action via the GHSR1a on AgRP neurons is partially responsible for its effect on food intake, but expression of GHSR also acts to normalize glycemia under fasted and food restricted conditions via effects on plasma glucagon and an upregulation of gluconeogenesis gene expression (105). Along with other similar findings on feeding and glycemia (16), these results suggest that ghrelin acts via the GHSR in AgRP neurons primarily to control glycemia in response to negative balance, with a secondary effect on feeding. Consistent with these physiological studies, the GHSR is expressed by a large majority of AgRP neurons (90%) and a significant portion of Growth hormone releasing hormone neurons (25%) and chemogenetic inhibition of GHSR neurons in the mediobasal hypothalamus blocks fasting-induced feeding, whereas chemogenetic activation increases food intake in satiated mice (15). Also highlighting the importance of the ghrelin-AgRP nexus is the ability of plasma ghrelin to rapidly enter the ARC for sensing by ARC (AgRP) neurons. In fact, this is the most prominent site for plasma ghrelin entry into the brain and accessibility increases during energy deficit (106–108). Taken together, these findings underline the important interaction between ghrelin and AgRP neurons that is magnified in situations of energy deficit such as AN. Indeed, AgRP neurons are required to integrate signals of energy status for the normal action of ghrelin, as we recently showed that glucose-sensing via AMPK in AgRP neurons modulates the ability of ghrelin to stimulate food intake (109).

AgRP neurons are important to sense and compute incoming information related to energy availability, a process that involves both sensory detection from olfactory and visual cues (12), as well as metabolic feedback in response to food consumption (110–112). Fiber photometry to visualize AgRP population activity showed a rapid reduction in fasted AgRP activity (within seconds) in response to the presentation of food, with a greater reduction in response to highly palatable foods (12). The reduction in AgRP activity was sustained only if food remained available for consumption after presentation and AgRP activity returned to high fasted levels if food was inaccessible or removed after presentation (12). Su et al. showed that

sustained reductions in AgRP neurons required gastro-intestinal nutrient and hormonal feedback over a longer timescale (30 min) (110, 111). These results demonstrate that AgRP neurons are responsive to different feedback modalities over different time frames—sensory feedback occurs within seconds and predicts the value of incoming nutrients, whereas nutrient and hormonal feedback occurs over minutes and provides a post-ingestive confirmation of actual calorie consumption to sustain changes in AgRP feedback. We recently showed that carnitine acetyltransferase (Crat) in AgRP neurons is an important enzyme required for the normal response to calorie intake during fasting, calorie restriction and restricted feeding (112–114), highlighting that normal metabolic processing of AgRP neurons is required to detect and compute calorie feedback.

Interestingly, signals of long term energy storage, such as leptin from adipose tissues, provides feedback to control AgRP neuronal activity over hours to days (110). Each aspect of the temporal feedback model may be important for normal homeostatic and behavioral actions of AgRP neurons and ghrelin, as a hormone that increases AgRP activity. If adipose stores are depleted, the absence of long-term feedback from leptin may affect both the sensory (seconds) and homeostatic (minutes) response to food. Indeed, AN is characterized by a loss of both long-term and homeostatic post-ingestive responses due to both the lack of food intake and absence of leptin, which has significant impact on the sensory control of AgRP. As a result, this may impair immediate behavioral and stress responses, something that is often reported in AN patients.

AgRP AND GHRELIN SIGNALING IMPACT ON BEHAVIOR

Optogenetic stimulation of hypothalamic axon terminals in the paraventricular hypothalamic nucleus, lateral hypothalamus, and in extra-hypothalamic axon terminals in bed nucleus of the stria terminalis, paraventricular thalamus, and medial amygdala increase food intake (8, 10, 17, 97, 99, 115). An intriguing observation is that there are a number of brain regions innervated by AgRP neurons that have no effect on food intake or other metabolic parameters (17). In addition, a number of the brain regions innervated by AgRP neurons that increase food intake also play important roles in the modulation of mood and motivation, including the output regions of the hypothalamus described above. Thus, AgRP neurons, as key neurons detecting hunger, are anatomically connected to numerous brain regions to control both feeding-related and non-feeding related behaviors.

Besides food intake, acute activation of AgRP neurons drives motivation to obtain food rewards, food-seeking locomotor behavior and a number of peripheral changes to limit energy expenditure (11, 63). In addition, AgRP neuronal activation is shown to evoke stereotypical behavioral patterns including repetitive obsessive and compulsive tendencies (9) when food was not available for consumption, similar to symptoms of AN. Optogenetic activation of AgRP neurons initiates a conditioned place aversion when food is not available, suggesting that increased motivation after AgRP neuronal activation is driven by the desire to remove the aversive feeling, otherwise known as negative reinforcement (11). Notably, fasting, ghrelin and AgRP activation all increase exploratory and risk-taking behavior in order to access food (7, 10, 116, 117). An important distinction here is that food is available during the task if the mouse is willing to risk obtaining it. Taken together, these data establish that AgRP neurons drive a neural signal of hunger, but if this neural signal of hunger is not fulfilled by appropriate food intake, or accessibility to food, this leads to non-feeding behaviors such as obsessive and compulsive tendencies and hyperlocomotion; that is, increased motivation driven by negative reinforcement. Such a response to hunger in the absence of food intake could underlie behavioral changes seen in AN, such as increased motivation for locomotion (exercise) rather than food (118).

Hunger-sensitive AgRP neurons and ghrelin feedback regulate non-food related behaviors, such as mood and motivation, which may be a result of an interaction between the ghrelin-AgRP nexus and stress pathways. This interaction can be bidirectional whereby fasting may activate the ghrelin-AgRP nexus to influence the Hypothalamo-Pituitary-Adrenal (HPA) stress axis (119, 120) or the HPA stress axis affecting the ghrelin-AgRP nexus (121). This interaction is pertinent, since AN patients show increased activation of the HPA stress axis at both the neuroendocrine (increased corticotropic-releasing hormone) and endocrine level (increased cortisol) (122–124), both of which are broadly implicated in neuropsychiatric disease (125). However, it should be noted that ghrelin can also directly activate corticotropic-releasing hormone neurons independently from the ARC (126, 127), indicating that behavioral changes associated with high ghrelin may simultaneously, yet independently, occur at the ARC and paraventricular hypothalamic nucleus.

Nevertheless, all psychological or physical stressors increase plasma ghrelin (128) and ghrelin regulates the HPA axis at the level of the pituitary and hypothalamus (129). The HPA axis mediates the body's response to stressors and facilitates the appropriate mechanisms to deal with stressful events (128). However, dysregulation of the HPA axis can prove maladaptive by promoting mood disorders, such as anxiety, depression, and compulsion (130), or metabolic disorders such as overeating and excessive weight gain (131, 132). In terms of regulating mood, GHSR signaling reduces anxiety and depression-like symptoms in a model of chronic social defeat (133, 134) and a Leu72Met gene polymorphism in the human *ghrelin* gene associates with major depression (135). In response to acute stress, ghrelin regulates the HPA axis to limit anxiety-like behavior (128, 129). However, this appears to be related to the ratio of acyl ghrelin to des-acyl ghrelin since mice lacking the enzyme that acylates ghrelin (GOAT) show increased anxiety-like behavior under both non-stressed and stressed conditions, which not due to changes in corticosterone (136). In addition, there is an unusual paradox, as a number of publications have reported that ghrelin promotes anxiety (137–139). In these studies, animals underwent behavioral testing within 30 min of ghrelin injection without food availability, suggesting that the unfulfilled hunger signal from ghrelin may have promoted an anxiety-like state during the testing period.

GHSR signaling in the brain also influences motivation for food rewards in models of conditioned place preference and operant conditioning (91, 140–143). It is particularly relevant that GHSR signaling in the brain may link stress/mood with the motivation to obtain food reward. For example, chronic social defeat stress in mice drove consumption of high fat diet and weight gain in GHSR wild-type but not GHSR knockout mice (131). Moreover, we have demonstrated that a ghrelin injection conditions a rewarding experience when paired with food availability but conditions an aversive experience when food is withheld (81), similar to examples above showing that AgRP neuronal activation in the absence of food drives a conditioned place aversion. Thus, plasma ghrelin, as a hunger signal from the body, influences mood and motivation and the behavioral readout depends on food availability.

How hunger states can affect mood and motivated behaviors needs addressing when we consider the co-morbidity between metabolic dysfunction and mental illness (144). Moreover, exactly where in the brain both metabolic and mood/motivation circuits interact remains unknown. One important region may be the amygdala given its roles in emotional learning, cue-predicted learning, anxiety, reward processing, and motivation (145). Indirect evidence shows that ghrelin regulates the activity of neurons in the medial amygdala after acute stress (38) and GHSR signaling in the basolateral amygdala regulates neuronal activity in a model of cue-potentiated feeding (146). Furthermore, repeated ghrelin agonist injections in the basolateral amygdala increased fear memory (147). In terms of AN, brain-imaging studies show differential activation of the amygdala in AN patients relative to controls (148) and homeostatic signals such as ghrelin, AgRP and NPY are all significantly increased in AN patients (149, 150). Interestingly, AN patients have significantly higher plasma ghrelin concentrations compared to constitutively lean women (151) and constitutional thinness is not associated with psychological disturbances, amenorrhea, or other hormonal abnormalities associated with undernutrition (36, 152). The mechanisms underlying this difference may be related to increased exercise often observed in AN patients, since exercise is known to increase plasma ghrelin concentrations (153). It is therefore plausible that persistent high levels of plasma ghrelin may contribute to mental health issues in AN patients.

AN patients have other behavioral maladaptations/ disturbances not apparently linked to hunger sensing (via

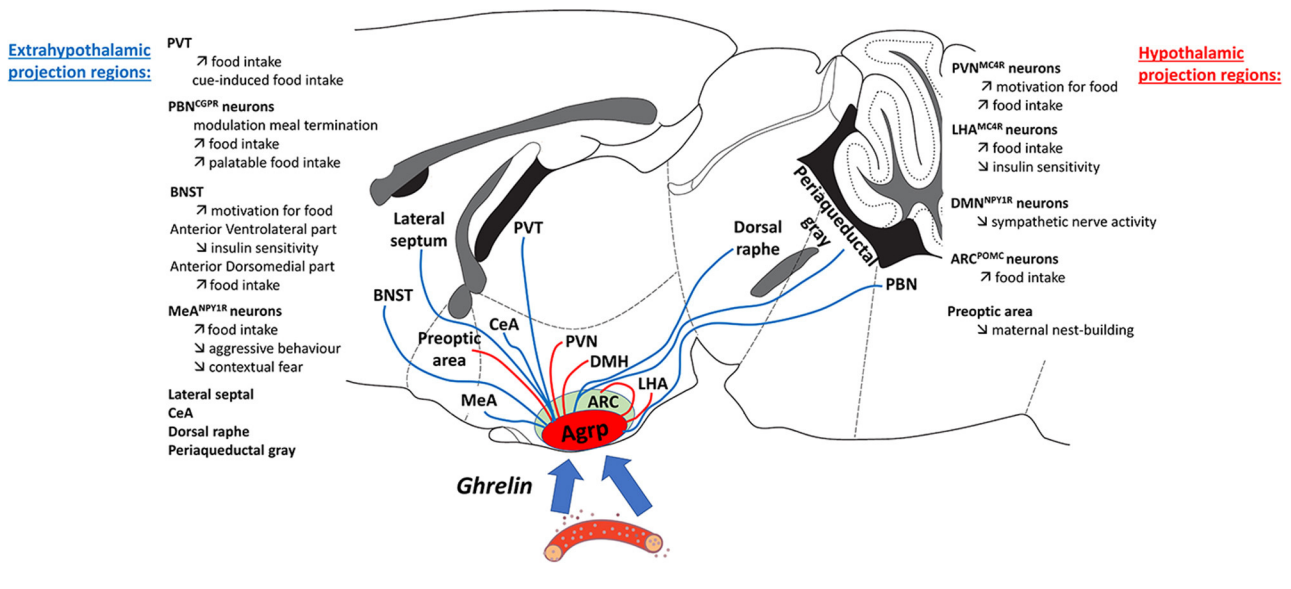

FIGURE 1 | Ghrelin is secreted from the stomach primarily under conditions of negative energy balance and acts to inform the brain of low energy availability. As a signal of energy deficit, ghrelin promotes behaviors to encourage food-seeking and food intake as well as adaptive strategies to cope with hunger, and influence metabolism to maximize energy storage. One of the major targets of circulating ghrelin is the population of AgRP neurons that reside in the arcuate nucleus of the hypothalamus. Ghrelin reaches AgRP neurons and fasting increases permeability to allow greater diffusion of ghrelin into this central target. As highlighted in this figure, AgRP neurons project to a large number of different nuclei throughout the hypothalamus, amygdala, brainstem, thalamus, and midbrain. However, not all AgRP neurons projections stimulate food intake, it is currently thought that only the Agrp to PVN, LHA, BNST, PVT, PBN, and MeA projections influence food intake. Thus, it is important to appreciate that activation of hunger-sensing AgRP neurons affects both feeding and nod feeding pathways when active. Another important observation is that ghrelin and fasting both increase AgRP neuron activity, leading to increased food intake when food is available; whereas when food is unavailable, the activation of AgRP neurons leads to changes in energy metabolism and behavioral adaptations. Such behavioral changes in the absence of food include obsession-compulsion, mood-changes, motivation, aversion, sociability, although the specific circuits involved in these behaviors remain to determined. In situations of acute energy deficit, these behavioral responses are thought to be adaptive, however the consequences of long-term energy deficit on these behavioral response remain unknown. These observations highlight a potential role for disrupted or prolonged chronic ghrelin-AgRP signaling in the absence of appropriate food intake to have a significant impact on normal behavior in anorexia nervosa (AN), a disorder characterized by a severe and chronic energy deficit. Indeed, similar behavioral features have also been observed in patients with AN, therefore, understanding how Ghrelin-AgRP neuronal signaling mediates behavioral and metabolic adaptations in the presence or absence food availability may shed light on the role of these circuits in the pathophysiology of AN.

AgRP neurons) or hunger signaling (via ghrelin or GHSR). These behaviors include disrupted sleep-wake structure and quality with lower slow wave sleep and rapid eye movement sleep, in addition to harm avoidance and social interaction deficits (154–156). Food restriction protocols in rodents are known to disturb the normal light-dark cycle activity in mice, as shown by food anticipatory activity and a recent study indicating that optogenetic AgRP neuronal activation increased the number and length of wake periods and the duration of non-rapid eye movement (NREM) sleep periods (157). Conversely, chemogenetic inhibition of these same neurons has no effect in satiated mice but reduced NREM sleep and microarousals during NREM sleep in fasted mice (157). Thus, persistently high AgRP and ghrelin levels as seen in AN (149, 150), may also impact behavior via impairing the quality of sleep.

CONCLUSION

An understanding of how hunger signals influence mood and motivation may provide valuable insight into the pathogenesis of both metabolic dysfunction and mental illnesses, such as AN. Indeed, AN is viewed as primarily a psychiatric disorder owing to the considerable behavioral and genetic overlap with mood disorders and other psychiatric traits (158). However, the recent reconceptualization of AN as one of both psychiatric and metabolic etiology (19, 20) suggests that metabolic circuits conveying hunger, or sensitive to signals of hunger, may be a critical nexus linking metabolic dysfunction to mood disturbances (see **Figure 1**). In line with this line of reasoning one would expect that dampening down persistent signals of hunger (AgRP neurons or GHSR activity) may alleviate some potential psychiatric problems associated with AN. However, this would be considered controversial and require substantial experimental evidence to support such actions.

The advent of new technologies developed this last decade has brought with it a new suite of information regarding the activity and function of AgRP neurons within hypothalamic and extrahypothalamic circuits. These neurons appear to be sensitive to a wide range of signals including food cues, nutrients and hormones and respond to these signals (8, 159). In light of this, it is clear that the AgRP neurons may have a significant role in AN at both a metabolic and behavioral level. Future studies are required to examine the causal role of hunger-sensing AgRP neurons and the hunger signal, ghrelin, in behavioral changes associated with AN. A major limitation at this stage, due to the complexity of the etiology of the disease, is an appropriate animal model in which to do so. Novel translational models should incorporate both voluntary reduction in food intake and excessive exercise behavior, both essential elements of body weight loss in AN, in addition to genetic, metabolic and environmental drivers of the human condition.

AUTHOR CONTRIBUTIONS

MM wrote the first draft of the manuscript. ZA wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SYSTEMATIC REVIEW
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White Matter Abnormalities in Anorexia Nervosa: Psychoradiologic Evidence From Meta-Analysis of Diffusion Tensor Imaging Studies Using Tract Based Spatial Statistics

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Background: Anorexia nervosa (AN) is a debilitating illness whose neural basis remains unclear. Studies using tract-based spatial statistics (TBSS) with diffusion tensor imaging (DTI) have demonstrated differences in white matter (WM) microarchitecture in AN, but the findings are inconclusive and controversial.

Objectives: To identify the most consistent WM abnormalities among previous TBSS studies of differences in WM microarchitecture in AN.

Methods: By systematically searching online databases, a total of 11 datasets were identified, including 245 patients with AN and 246 healthy controls (HC). We used Seed-based d Mapping to analyze fractional anisotropy (FA) differences between AN patients and HC, and performed meta-regression analysis to explore the effects of clinical characteristics on WM abnormalities in AN.

Results: The pooled results of all AN patients showed robustly lower FA in the corpus callosum (CC) and the cingulum compared to HC. These two regions preserved significance in the sensitivity analysis as well as in all subgroup analyses. Fiber tracking showed that the WM tracts primarily involved were the body of the CC and the cingulum bundle. Meta-regression analysis revealed that the body mass index and mean age were not linearly correlated with the lower FA.

Conclusions: The most consistent WM microstructural differences in AN were in the interhemispheric connections and limbic association fibers. These common "targets" advance our understanding of the complex neural mechanisms underlying the puzzling symptoms of AN, and may help in developing early treatment approaches.

Keywords: anorexia nervosa, diffusion tensor, tract-based spatial statistics, fractional anisotropy, magnetic resonance imaging, psychoradiology

INTRODUCTION

Anorexia nervosa (AN) is a serious mental and somatic disorder that typically develops during adolescence and primarily affects females (Zipfel et al., 2015). With a prevalence of about 0.3% it is relatively rare, but has serious medical consequences leading to death in ∼10% of cases, and thus poses a major clinical, psychological and societal burden (Nielsen, 2001). AN is characterized by extreme restriction of energy intake, a distorted body image, excessive concerns over weight and shape, and emotional dysfunction (American Psychiatric Association (APA), 2013; Zipfel et al., 2015). There may be severe long-term medical and psychological sequelae besides acute effects of self-starvation (Steinhausen, 2002). The etiology of AN remains unknown, and the interaction of neurobiological, psychological and environmental factors in its onset and outcome is unclear (Kaye et al., 2013; Zipfel et al., 2015). Exploring the neurobiological abnormalities associated with AN will be important for improving the effectiveness of both diagnosis and treatment (Hill et al., 2016).

With the development of noninvasive neuroimaging technology, diffusion tensor imaging (DTI), as an important psychoradiologic technique (Lui et al., 2016; Kressel, 2017; Port, 2018; Sun et al., 2018; https://radiopaedia.org/articles/psychoradiology), has become a powerful tool for detecting white matter (WM) microstructural differences in various psychiatric illnesses, including schizophrenia (Hao et al., 2006), depression (Kieseppa et al., 2010) as well as bipolar disorder (Wessa et al., 2009). Fractional anisotropy (FA) is the most commonly used DTI metric for exploring anisotropy, quantifying the directionality of diffusion. FA is considered as a highly sensitive but fairly non-specific biomarker of brain WM microstructural architecture and neuropathology (Alexander et al., 2007).

To investigate whole brain FA differences, metrics can be extracted globally by either voxel-based analysis (VBA) or tract-based spatial statistics (TBSS). Several such studies have demonstrated FA differences between patients with AN and healthy controls (HC). Unfortunately, their results are not consistent. Most studies report *lower* FA in widespread WM regions, including the corpus callosum (CC) (Frieling et al., 2012; Frank et al., 2013; Shott et al., 2016; Gaudio et al., 2017; Phillipou et al., 2018; von Schwanenflug et al., 2019), fornix fibers (Kazlouski et al., 2011; Frank et al., 2013; Gaudio et al., 2017), thalamus (Frieling et al., 2012; Hu et al., 2017), cingulum (Kazlouski et al., 2011; Frank et al., 2013), posterior thalamic radiation (PTR) (Phillipou et al., 2018), superior longitudinal fasciculus (SLF) (Via et al., 2014), fronto-occipital fasciculus (FOF) (Kazlouski et al., 2011; Via et al., 2014), corona radiation (Shott et al., 2016; Phillipou et al., 2018) and cerebellum (Nagahara et al., 2014; Shott et al., 2016). Five studies, however, observed *no* significant FA differences between AN patients and HC (Yau et al., 2013; Cha et al., 2016; Pfuhl et al., 2016; Bang et al., 2018; Olivo and Swenne, 2019). Two studies also reported *higher* FA in corona radiation, SLF, FOF, PTR, and CC (Frank et al., 2013; Vogel et al., 2016). These inconsistencies might be due to differences in sample size or in the demographic and clinical characteristics of the patients, and heterogeneity in the imaging protocols. In such situations a powerful way to isolate reliable neurobiological markers is meta-analysis.

To our knowledge, only Barona and colleagues have conducted a coordinate-based meta-analysis of whole-brain DTI studies in AN (Barona et al., 2019). However, the study has a major limitation in using two different methods (TBSS and VBA) to undertake whole brain analysis. VBA is relatively direct, involving spatial normalization of high-resolution images from all subjects to the same stereotactic space (Ashburner and Friston, 2000). By contrast TBSS is a statistical approach, particularly developed to analyze DTI data. It restricts analysis to the center of major WM fibers by projecting every subject's FA data onto the mean skeleton, thus alleviating the misalignment problems that can affect regular VBA. Briefly, TBSS is a more accurate method for exploring disorganization of WM architecture (Smith et al., 2006).

Our aims in this paper are: first, to conduct an updated meta-analysis of TBSS studies to define the most prominent and replicable WM microarchitecture abnormalities in patients with AN using Seed-based d Mapping (SDM), a statistical technique for meta-analyzing studies which use neuroimaging techniques such as fMRI, VBM, DTI or PET to investigate the changes of brain activity or structure (https://www.sdmproject.com/). This method is now widely accepted and has been used in studies of major depressive disorder (Jiang et al., 2017), childhood maltreatment (Lim et al., 2014) and bipolar disorder (Wise et al., 2016). Second, to perform subgroup meta-analyses based on the effects of age and stage of the disorder. Third, to use meta-regression to examine the potential effects of age, illness duration and body mass index (BMI) on the reported WM abnormalities. We hypothesized that AN patients would manifest lower FA compared to HC in tracts involved in reward-related processing (*viz.* CC, fornix, thalamic projections, and striatum) and limbic regions. We also speculated that WM microarchitecture abnormalities might be associated with factors related to starvation (*viz.* decreased BMI and illness duration) and AN symptomatology.

MATERIALS AND METHODS

Literature Search Strategy

We searched for publications on the PubMed, Ovid databases, Web of Science, Science Direct and Google Scholar. The last screen was performed in March 2019. The key search terms were: ("anorexia nervosa" or "eating disorder" or "anorexia") and ("tract-based spatial statistical" or "TBSS" or "diffusion tensor" or "DTI" or "diffusion tensor imaging" or "fractional anisotropy" or "FA"). The reference lists of identified studies and relevant reviews were manually checked for further studies.

Selection Criteria and Data Extraction

Studies were included according to the following criteria: (a) articles written in the English language and published in peer-reviewed journals; (b) a primary diagnosis of AN according to the international classification of diseases-10 (ICD-10) and/or

Diagnostic and Statistical Manual of Mental Disorders (DSM); (c) studies reported a TBSS comparison between patients with AN and HC; (d) studies detected FA differences at the whole-brain level and reported the results in stereotactic 3D coordinates (Talairach or MNI). When details were not reported in the original manuscripts, a request was made to the corresponding author by e-mail.

Studies were excluded according to the following criteria: (a) meta-analysis, case reports or reviews; (b) studies lacking a HC group; (c) if several studies reported overlapping samples, only the paper reporting the largest sample size was selected. We conducted this meta-analysis according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (Liberati et al., 2009).

The quality of each included study was assessed using a 12-point checklist (see **Table S1**) that focused on both the clinical and demographic aspects of individual studies and on the imaging methodology (Du et al., 2014). From each included study we recorded first author, cohort size, demographics (age and gender), illness variables (stage of AN, subtype of AN, age at onset, illness duration, BMI, symptom severity), imaging parameters, data processing method and statistical threshold; the peak coordinates were extracted using the SDM tool (Radua et al., 2014b). Two authors (SZ and WW) did this independently, any disagreement being resolved by discussion.

SDM Meta-Analysis

We conducted a voxel-based analysis to identify brain regions showing consistent significant differences in FA between AN patients and HC, according to the standardized process of the SDM software (http://www.sdmproject.com). Briefly, the SDM tool recreates a map of the effect size based on the peak coordinates extracted from each included study.

The robustness of the main findings was checked by three complementary analyses. First, jack-knife sensitivity analysis was performed to assess the replicability of the results by iteratively repeating the same analysis, discarding a data set each time to establish whether the results remained significant (Radua et al., 2014a). Second, a random-effects model with Q statistics was used to detect the statistical (between-studies) heterogeneity of individual clusters. Third, Egger tests using STATA (www.stata.cn) were used to assess publication bias.

Initially, we planned to perform subgroup meta-analysis of adolescent vs. adult subjects, medicated vs. drug-free subjects, as well as acute cases vs. recovered subjects. However, the number of studies in most of these subgroups (adolescent, medicated, drug-free, recovered) was too small to draw reliable conclusions. Finally, meta-analysis of the subgroups was conducted only for the adult subjects and the acute subjects.

All analytical processes were as described in the SDM tutorial (https://www.sdmproject.com/software/tutorial.pdf) and related publications (Radua et al., 2014b). We adopted the default SDM thresholds (anisotropy = 1.0; full-width at half-maximum = 20 mm, voxel $p = 0.005$, peak height threshold $Z = 1$, cluster extent = 10 voxels) (Radua et al., 2012).

To convert the SDM results into images, we used MRIcron software (http://www.mricro.com/mricron/), and overlaid the results on a high-resolution brain image template (created by the International Consortium for Brain Mapping) and the FMRIB58_ FA skeleton.

We then used DSI Studio to identify and visualize the WM tracts most probably involved, working as described in the DSI

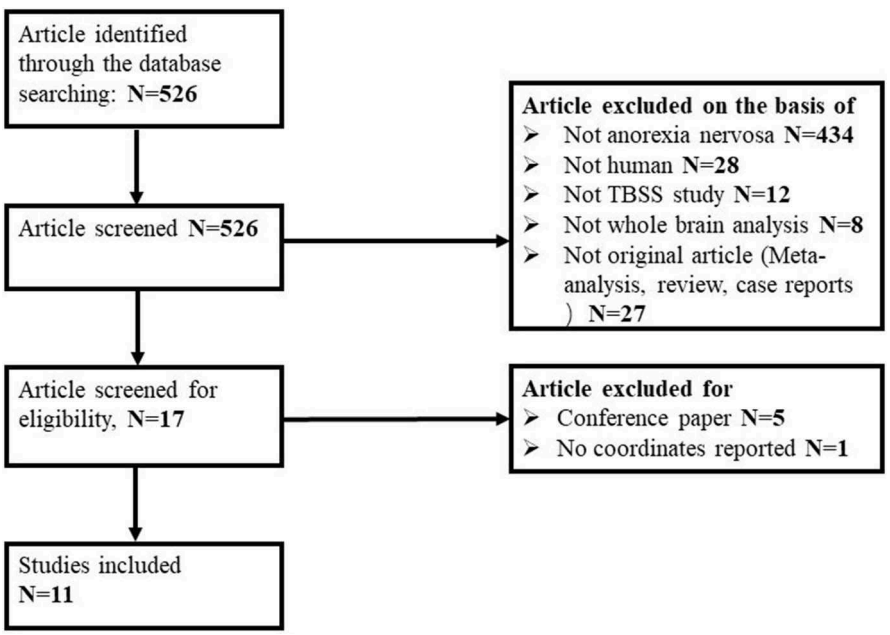

FIGURE 1 | Flow diagram for the identification and exclusion of studies.

studio tutorial (http://dsi-studio.labsolver.org). Meta-analysis results were projected onto a high-resolution diffusion magnetic resonance imaging dataset generated from 80 subjects of the Human Connectome Project (Van Essen et al., 2012). A three-dimensional atlas of human white matter tracts (Catani and Thiebaut de Schotten, 2012) was used to identify the implicated tracts.

Meta-Regression Analysis

Clinical variables explored by meta-regression analyses were mean BMI, age, Beck Depression Inventory (BDI) and illness duration, and percentages of females, and medicated patients. As in previous meta-analyses (Jiang et al., 2017) and in accordance with the recommendations of SDM's authors (Radua and Mataix-Cols, 2009), we adopted a conservative threshold of $p = 0.0005$ to minimize Type I error.

RESULTS

Description of Included Studies

Of 526 potentially relevant studies, 11 met our criteria, as summarized in **Figure 1**. The 11 included studies recruited a total of 245 AN patients and 246 HC. **Table 1** summarizes the clinical and demographic data from all included studies. The clinical characteristics (age, sex) of these studies showed no

TABLE 1 | Demographic and clinical characteristics of the participants in the 11 studies on anorexia nervosa included in the meta-analysis.

Study	AN stage	AN subtype	Number (female)		Age (y)		Duration	BMI		BDI score	Onset age (y)	Medication %	Comorbidity
			AN	HC	AN	HC	(y)	AN	HC				
Yau et al. (2013)	REC	R	12 (12)	10 (10)	28.7	26.7	5.6	21.2	22.0	NA	15.5	Drug-free	No
Nagahara et al. (2014)	A	R and B/P	17 (17)	18 (18)	23.8	26.2	4.93	13.6	19.9	30.2	NA	35	Yes
Via et al. (2014)	A	R	19 (19)	19 (19)	28.3	28.6	6.52	17.0	21.1	NA	21.8	26	Yes
Shott et al. (2016)	REC	R	24 (24)	24 (24)	30.3	27.4	5.90	20.8	21.6	NA	16.7	25	Yes
Cha et al. (2016)	A	R and B/P	22 (22)	18 (18)	19.5	20.5	NA	17.3	21.2	NA	NA	Drug-free	Yes
Olivo et al. (2017)	A	EDNOS	12 (12)	14 (12)	15.3	14.1	NA	18.7	20.6	NA	NA	Drug-free	Yes
Bang et al. (2018)	REC	R and B/P	21 (21)	21 (21)	27.6	26.1	2.83	20.5	21.8	6.6	17.3	14	No
Gaudio et al. (2017)	A	R	14 (14)	15 (15)	15.7	16.3	0.41	16.2	21.1	30.4	15.4	Drug-free	No
Phillipou et al. (2018)	A	R and B/P	23 (23)	26 (26)	22.0	22.6	5.35	16.7	22.8	NA	16.4	NA	Yes
von Schwanenflug et al. (2019)	A	R	56 (56)	56 (56)	15.9	16.2	1.21	14.7	20.6	21.2	NA	2	No
Olivo and Swenne (2019)	A	Atypical	25 (25)	25 (25)	14.8	14.5	0.7	18.6	20.0	NA	NA	Drug-free	No

A, accurate; AN, anorexia nervosa; BDI, Beck Depression Inventory; BMI, body mass index; B/P: binge and purge subtype of anorexia nervosa; HC, healthy controls; NA, not available; R, restrictive subtype of anorexia nervosa; REC, recovered; EDNOS, eating disorder not otherwise specified.

TABLE 2 | Technical details of the 11 studies on anorexia nervosa included in the meta-analysis.

Study	MRI scanner	No. of DTI directions	Coordinate system	Analysis software	Analysis method	*p*-value	No. of coordinates
Yau et al. (2013)	3.0T	55	MNI	FSL	TBSS	$p < 0.05$ (FWE)	0
Nagahara et al. (2014)	3.0T	32	MNI	FSL	TBSS	$p < 0.08$ (corrected)	1
Via et al. (2014)	1.5T	25	MNI	FSL	TBSS	$p < 0.05$ (FWE)	1
Shott et al. (2016)	NA	25	MNI	FSL	TBSS	$p < 0.05$ (FWE)	6
Cha et al. (2016)	1.5T	16	MNI	FSL	TBSS	$p < 0.05$ (FDR)	0
Olivo et al. (2017)	3.0T	48	MNI	FSL	TBSS	$p < 0.05$ (FDR)	2
Bang et al. (2018)	3.0T	32	MNI	FSL	TBSS	$p < 0.05$ (FWE)	0
Gaudio et al. (2017)	1.5T	12	MNI	FSL	TBSS	$p < 0.05$ (FWE)	4
Phillipou et al. (2018)	3.0T	60	MNI	FSL	TBSS	$p < 0.05$ (FWE)	1
von Schwanenflug et al. (2019)	3.0T	32	MNI	FSL	TBSS	$p < 0.05$ (FWE)	1
Olivo and Swenne (2019)	3.0T	48	MNI	FSL	TBSS	$p < 0.05$ (TFCE)	0

DTI, diffusion tensor imaging; FDR, false discovery rate; FSL, functional MRI of the brain (FMRIB) software library; FWE, family-wise error; MNI, Montreal Neurological Institute Space; NA, not available; TBSS, tract-based spatial statistical; TFCE, threshold-free cluster enhancement.

differences between AN and HC groups. **Table 2** summarizes technical details of all included studies.

Meta-Analysis

Pooled Voxel-Based Meta-Analysis

As illustrated in **Figure 2** and **Table 3**, the pooled meta-analysis revealed significantly *lower* FA in AN patients relative to HC in two regions: CC and cingulum. No regions showed *higher* FA. As shown in **Figure 3**, the WM tracts mainly involved were the cingulum bundle and the interhemispheric fibers running through the CC.

Subgroup Voxel-Based Meta-Analysis

The adult AN subgroup included seven datasets that showed *lower* FA in the CC and cingulum bundle, sharing same clusters with the pooled meta-analysis. No regions showed *higher* FA in adult AN (**Table 4**).

The acute AN patient subgroup included 8 datasets that showed *lower* FA in the CC and cingulum bundle. No regions showed *higher* FA in AN (**Table 4**). These results are consistent with the pooled meta-analysis, indicating that the main effects related to the acute AN patients rather than recovered AN.

Reliability Analysis

The whole-brain jack-knife sensitivity analysis showed that *lower* FA in the CC and cingulum was highly reliable, being retained throughout 10 datasets combinations (**Table 3**). Analysis of heterogeneity revealed that the CC and cingulum with lower FA had significant statistical heterogeneity among studies ($p < 0.005$) (**Table 3**). Analysis of publication bias by the Egger test was non-significant for CC ($p = 0.137$) and cingulum ($p = 0.484$) (**Table 3**).

Meta-Regression Analysis

Mean age and BMI showed no relationship with lower FA. Illness duration, BDI score and percentage of medicated patients could not be examined because of limited data. AN symptom severity could not be examined because it was reported using various inconsistent measures.

DISCUSSION

This is the first quantitative meta-analysis integrating TBSS studies in patients with AN. Partly consistent with our hypotheses, pooled analysis revealed that the most robust disruption of WM microstructure, reflected in lower FA, in AN patients were in the CC and cingulum. Subgroup analyses of adult studies and acute studies replicated these findings. However, we found no significant correlations between BMI and lower FA.

Lower Fractional Anisotropy in the Corpus Callosum and Cingulum Bundle

The biggest cluster with lower FA in AN was the CC, as reported in several studies (Frieling et al., 2012; Frank et al., 2013; Shott et al., 2016; Gaudio et al., 2017; Olivo et al., 2017; Phillipou et al., 2018; Barona et al., 2019; von Schwanenflug et al., 2019). The CC is the largest interhemispheric commissure, communicating perceptual, cognitive, motor and affective information (Hofer and Frahm, 2006; Catani and Thiebaut de Schotten, 2008).

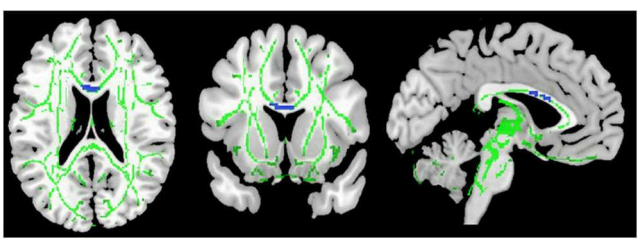

FIGURE 2 | Results of pooled meta-analysis. Regions with blue color show lower FA in AN patients compared with healthy controls in the CC. FA, fractional anisotropy; AN, Anorexia nervosa; CC, Corpus callosum.

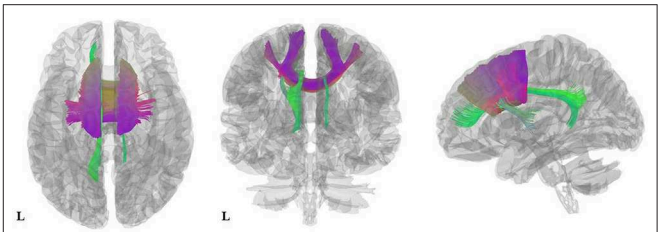

FIGURE 3 | Results of pooled meta-analysis. Three-dimensional images show the most probable white matter tracts running through CC (purple) and the cingulum (green) bundle in AN patients. AN, Anorexia nervosa; CC, Corpus callosum.

TABLE 3 | Regions of lower fractional anisotropy in anorexia nervosa patients compared with health controls identified by the main meta-analyses.

Region	Maximum				Cluster	Robustness		
	MNI coordinates x, y, z	SDM z-score	*P*-value uncorrected	Number of voxels	Breakdown (No. of voxels)	Jackknife	Heterogeneity	Publication bias
Corpus callosum	0, 6, 24	−2.070	~0	289	Corpus callosum (275) Cingulum (14)	10 of 11	0.00001	ns

MNI, Montreal Neurological Institute Space; SDM, Seed-based d Mapping; ns, non-significant; Jackknife: The jackknife sensitivity analysis column gives the number of studies whose omission does not affect the finding.

TABLE 4 | Regions of lower fractional anisotropy in anorexia nervosa patients compared with health controls identified by the subgroup meta-analyses (acute subgroup; adult subgroup).

Region	Maximum				Cluster	Jackknife sensitivity analysis
	MNI coordinates x, y, z	SDM z-score	*P*-value uncorrected	Number of voxels	Breakdown (No. of voxels)	
Acute subgroup						
Corpus callosum	−6, 16, 20	−2.112	0.000024557	284	Corpus callosum (270) Cingulum (14)	8 of 8
Adult subgroup						
Corpus callosum	−6, 14, 22	−1.071	0.000147164	138	Corpus callosum (127) Cingulum (11)	6 of 7

MNI, Montreal Neurological Institute Space; SDM, Seed-based d Mapping.

Notably, the WM fibers crossing through the body of the CC connect the bilateral prefrontal cortices and supplementary motor areas (SMA), and microstructural alterations in the body of the CC, as reflected by the decreased FA, might lead to reduced quantity and speed of information transfer between these brain areas. The prefrontal cortices are involved in the affective element of body image, which can be conceptualized as feelings and the satisfaction or dissatisfaction with the body (Gaudio and Quattrocchi, 2012). Therefore, the lower FA in the body of CC in AN might reflect an impaired prefrontal interhemispheric connectivity, underlying or contributing to body image distortion in AN (Gaudio et al., 2014; Gadsby, 2017). The SMA is involved in the planning and control of motor actions, and plays an important role in task switching, especially in proactive behavioral switching (Nachev et al., 2008; Hikosaka and Isoda, 2010). Functional MRI has shown that the SMA is consistently activated when subjects switch between two tasks proactively in response to a cue (Rushworth et al., 2002). Therefore, we speculated that impaired WM integrity in the bilateral SMA might lead to cognitive-behavioral inflexibility (i.e. stereotyped or perseverative behaviors), which may contribute to behaviors for self-induced starvation. Furthermore, the observation that higher FA in the body of CC is positively correlated with reward-related activation in the nucleus accumbens suggests that CC might influence reward responsiveness of the ventral striatum by regulating the efficiency of information transfer within reward-related circuitries (Koch et al., 2014).

We also identified lower FA in the cingulum, in line with prior studies (Kazlouski et al., 2011; Frank et al., 2013). The cingulum incorporates fibers of different length: the longest running from the anterior temporal gyrus to the orbitofrontal cortex, while short *U*-shaped fibers link the medial frontal, parietal, occipital, and temporal lobes and different parts of the cingulate cortex (Catani and Thiebaut de Schotten, 2008). The cingulum is a component of the limbic system, involved in attention, memory and emotions (Catani, 2006; Rudrauf et al., 2008). Given that the cingulum bundle is a key part of the network integrating behaviors necessary for emotion identification and processing (Kazlouski et al., 2011), disruption of WM microstructures in this area could explain abnormalities in emotion recognition and regulation in AN, such as difficulties in concentrating and accomplishing tasks when experiencing negative emotions (Harrison et al., 2010).

Interestingly, these results of lower FA in the CC and cingulum were retained in the subgroup meta-analysis. The findings seem to show that the CC and cingulum are stable markers of the disorder and interruptions in WM tracts of these areas may be involved in the pathological mechanisms of AN. As the numbers of studies in the subgroup meta-analyses are relatively small (seven and eight respectively), we should treat these results with caution. Additionally, because limited data precluded meta-analysis of the recovered AN group, whether or not the alterations persist after recovery is a question still to be addressed.

Null Results by Meta-Regression Analysis

Although there were no significant associations between clinical variables and WM abnormalities, the effect of self-starvation (*viz.* decreased BMI) is particularly interesting. Previous studies have variously reported significant correlations (Kazlouski et al., 2011; Nagahara et al., 2014; Olivo et al., 2017) and no correlations (Gaudio et al., 2017; Bang et al., 2018; Phillipou et al., 2018) between BMI and FA in different brain areas. Heterogeneity in patient characteristics may contribute to this negative result. Alternatively, it may indicate that WM microstructure impairments in AN are not directly related to effects of starvation, but instead to trait characteristics of the disorder (Phillipou et al., 2018). Nevertheless, these preliminary findings need to be validated by longitudinal studies.

An Unexpected Lack of Abnormality

Compared with a previous meta-analysis of AN, which revealed disturbed WM in various regions (e.g. clusters with lower FA in the left superior longitudinal fasciculus and left precentral gyrus, and higher FA in the right cortico-spinal projections and lingual gyrus) (Barona et al., 2019), the present meta-analysis predominantly emphasized interhemispheric communication and the limbic association fibers. This inconsistency might be explained in two ways. Firstly, we only analyzed DTI studies using TBSS, not VBA, thus avoiding any bias arising

from methodological differences in diffusion data processing. Secondly, we included a number of new studies, with resulting differences in sample characteristics (e.g., age, gender, subtype, and medication status).

Limitations of This Study

The study has some limitations. Firstly, voxel-based meta-analyses are based on summarized data (i.e. coordinates and effect sizes from published studies). Although analyzing a cumulative set of primary data would in theory yield more accurate results, it is rarely feasible to obtain raw image files. Secondly, we could not take AN-subtypes into consideration; the restricting subtype and the binge-purging subtype may have different etiologies, but this was hard to explore because the information was not available. Thirdly, although we found the lower FA in the CC and cingulum retained significance in adult AN and acute AN subgroup analyses, it cannot be concluded that these abnormalities are a biomarker of the disorder, since the differences in their comparator groups (recovered AN and adolescent AN) are still unknown. More studies on these subgroups are needed. Fourthly, the mean FA skeleton is different in each study due to heterogeneity in the data, which may decrease the accuracy of the results of the meta-analysis. Finally, it is useful to combine FA with other diffusion parameters (MD, AD, and RD); unfortunately, most of the included studies did not report them.

CONCLUSION

This meta-analysis detected significantly lower FA in AN in the WM of the interhemispheric connection and limbic association fibers, which are involved in body cognitive-behavioral inflexibility, image processing and emotional function. Although the neuropathology of AN is complex, our findings help provide evidence on how symptoms and behaviors are encoded in the brain, and thus may aid in developing effective treatments. Future studies with a longitudinal approach are needed to confirm our results and to reveal the trajectory of the pathophysiology.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

QG and QY conceived the project. HS designed the protocol. SZ and WW wrote the main manuscript. SZ, WW, XS, XY, JS, and QT obtained the data. SZ, LL, and YZ analyzed the results. All authors critically reviewed the manuscript. GK, QG, and QY revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00159/full#supplementary-material

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