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In pursuit of biomarkers for predicting susceptibility to activity-based anorexia in adolescent female rats

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Abstract

Objective: Identifying risk factors that contribute to the development of anorexia nervosa (AN) is critical for the implementation of early intervention strategies. Anxiety, obsessive–compulsive behavior, and immune dysfunction may be involved in the development of AN; however, their direct influence on susceptibility to the condition remains unclear. Here, we used the activity-based anorexia (ABA) model to examine whether activity, anxiety-like behavior, compulsive behavior, and circulating immune markers predict the subsequent development of pathological weight loss.

Method: Female Sprague–Dawley rats ($n = 44$) underwent behavioral testing before exposure to ABA conditions after which they were separated into susceptible and resistant subpopulations. Blood was sampled before behavioral testing and after recovery from ABA to screen for proinflammatory cytokines.

Results: Rats that were vulnerable to pathological weight loss differed significantly from resistant rats on all key ABA parameters. While the primary measures of anxiety-like or compulsive behavior were not shown to predict vulnerability to ABA, increased locomotion and anxiety-like behavior were both associated with the *extent of* weight loss in susceptible but not resistant animals. Moreover, the change in expression of proinflammatory markers IL-4 and IL-6 evoked by ABA was associated with discrete vulnerability factors. Intriguingly, behavior related to risk assessment was shown to predict vulnerability to ABA.

Discussion: We did not find undisputable behavioral or immune predictors of susceptibility to pathological weight loss in the ABA rat model. Future research should examine the role of cognition in the development of ABA, dysfunction of which may represent an endophenotype linking anorectic, anxiety-like and compulsive behavior.

Public Significance: Anorexia nervosa (AN) has among the highest mortality rates of all psychiatric disorders and treatment options remain limited in their efficacy. Understanding what types of risk factors contribute to the development of AN is essential for implementing early intervention strategies. This study describes how some of the most common psychological features of AN could be used to predict susceptibility to pathological weight loss in a well-established animal model.

KEYWORDS
anorexia nervosa, anxiety disorders, biomarkers, cytokines, disease models (animal), immune system, obsessive–compulsive disorder, predisposing factors, weight loss

1 | INTRODUCTION

Anorexia nervosa (AN) is a devastating condition with a mortality rate among the highest of any psychiatric disorder (Arcelus et al., 2011). Up to half of patients with AN never recover (Zipfel et al., 2000), highlighting the urgent need for more effective therapeutic strategies. Recovery from AN becomes much less likely the longer the illness has persisted (Von Holle et al., 2008); therefore, early detection of symptoms and intervention are necessary for improving treatment outcomes (Le Grange & Loeb, 2007). Identifying biomarkers to help predict AN risk and/or symptom severity would enable treatment to be directed at prodromal symptoms, before weight loss becomes too severe and protracted. Both comorbid diagnoses of and symptoms related to depression, anxiety, and obsessive–compulsive disorder (OCD) are associated with worsened illness, poorer treatment outcomes, and increased relapse in patients with AN (Keski-Rahkonen et al., 2014; Mischoulon et al., 2010; Thornton et al., 2011; Wild et al., 2016; Zerwas et al., 2013). AN often follows a pre-existing diagnosis of comorbid OCD, generalized anxiety disorder (GAD), and social phobia (Godart et al., 2000; Kaye et al., 2004; Meier et al., 2015; Strober et al., 2007), presenting potential targets for early intervention strategies. In addition, childhood traits including "picky eating", perfectionism, harm avoidance, and rigid patterns of thought and behavior (Anderluh et al., 2003; Degortes et al., 2014), related to levels of anxiety and compulsiveness (Kaye et al., 2004), have been associated with the subsequent development of AN. However, a diagnosis of AN can also precede the development of, or be determined coincident with, comorbid psychiatric disorders (Godart et al., 2015, 2007; Kaye et al., 2004), making it difficult to dissect the nature of the interaction between AN and depressive, anxious, and obsessive/compulsive symptoms. Rates of depression, anxiety, and OCD are higher in healthy first-degree relatives of patients with AN compared to the general population (Bellodi et al., 2001; Strober et al., 2007, 1990) and there is substantial genetic overlap between AN and generalized anxiety disorder (Dellava et al., 2011), major depressive disorder (Wade et al., 2000), and OCD (Yilmaz et al., 2020). Taken together, these findings suggest the existence of heritable risk factors for multiple psychiatric diagnoses that cluster together and sometimes result in the development of AN.

While association of mood disorders and AN as outlined above are often established in a post hoc manner, physiological correlates should provide earlier and more definitive predictors of subsequent susceptibility to AN. In this regard, disturbances in immune function may play a causal role in the development of AN, considering that children and adolescents with autoimmune and autoinflammatory disorders have been shown to have a higher risk of subsequent AN diagnoses (Zerwas et al., 2017) and genes related to various immune-related phenotypes are associated with AN (Duncan et al., 2017). Patients with AN have a distinct pattern of immune mediator expression, the most consistently documented of which are increased levels of the inflammatory markers RANTES (regulated upon activation, normal T cell expressed, and secreted) (Pisetsky et al., 2014), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) (Dalton et al., 2018). Proinflammatory cytokines can have profound effects on brain function and behavior relevant to food intake (Wong & Pinkney, 2004) but there is conflicting evidence about whether these remain elevated after weight recovery in AN (Nilsson et al., 2020; Solmi et al., 2015). It may be the case that at least some of these changes are consequences of the conditions of chronic malnutrition typical of AN; however, increased levels of the same proinflammatory cytokines (IL-6 and TNF-α) are seen in patients with OCD (Karagüzel et al., 2019) and generalized anxiety disorder is also associated with an inflammatory response that includes increased TNF-α (Costello et al., 2019). These conditions are not associated with chronic undernutrition but, as described above, are both commonly comorbid with AN, raising the potential that there are common inflammatory mechanisms involved in the etiology of these disorders.

In order to dissect behavioral and immune factors that predict the development of AN, animal models are required. The most robust and widely used animal model of AN is known as activity-based anorexia (ABA), which allows for a detailed interrogation of the biological mechanisms underlying pathological weight loss. The ABA model pairs unhindered access to running wheels with time-limited food access and recapitulates the core features of the human condition including rapid body weight loss, voluntary hyperactivity, and voluntary reductions in food intake (Gutierrez, 2013; Schalla & Stengel, 2019; Scharner & Stengel, 2021). We have previously shown that pathological weight loss does not occur in all rats exposed to ABA conditions, and in fact a subpopulation of rats remains resistant to weight loss when exposed to the same experimental conditions (Milton et al., 2018). These susceptible and resistant subpopulations have been replicated in other studies investigating the neurobiology of AN in ABA rats (Hurley, Murlanova, et al., 2021) and mice (Beeler et al., 2020), supporting the use of the ABA model for examining predictors of pathological weight loss.

Exposure to ABA conditions is shown to increase anxiety-like behavior on the elevated plus maze (EPM) and the open field (OF) test (Chen et al., 2017; Kinzig & Hargrave, 2010); however, much like human studies, it is unclear whether this is a *consequence* of weight loss or involved in vulnerability to ABA. Anxiety-like behavior has been examined after only a single day of restricted food access in ABA mice, prior to excessive weight loss, and shown to be negatively correlated with excessive wheel running but not associated with weight loss (Wable et al., 2015). Only one study to date has examined how immune function is altered in the ABA model, with increased expression of interleukin-10 (IL-10), TNF-α and interleukin-1β (IL-1β) observed in the colonic mucosa of ABA mice compared to mice subject to the same food restriction paradigm without access to a wheel, indicating an effect specific to ABA rather than the

result of limited food intake (Belmonte et al., 2016). The increased expression was accompanied by elevated levels of IL-1β and its receptor (interleukin-1 receptor-1 [IL-1R1]) in the hypothalamus of both ABA mice and food restricted controls and increased circulating zonulin, which modulates the permeability of tight junctions in the intestine. However, it remains unknown whether immune disturbances might predispose animals to developing the ABA phenotype.

In this study, we aimed to determine whether levels of anxiety-related and compulsive behavior in rats could be used to predict susceptibility to pathological weight loss under ABA conditions, and whether differences in proinflammatory cytokine expression correlated with behavior or the development of ABA. Our goal was to identify markers for the development of anorectic behavior in a well-established animal model that could be used to screen at-risk individuals in order to direct early intervention strategies. This objective is in keeping with broader efforts to define predictive markers of future susceptibility to ABA in rodents and AN in humans that encompass not only behavioral but also biological traits.

2 | METHODS

2.1 | Animals

Due to the higher prevalence of AN in adolescent women, female Sprague–Dawley rats ($n = 44$; 6 weeks old) were bred at the Monash Animal Research Platform (MARP) and transported to the behavioral testing suite 1 week prior to the commencement of experiments at which initial body weights were between 120-160 g. To determine whether baseline locomotor activity, anxiety-like, or compulsive behavior predicted susceptibility to weight loss in ABA, rats were tested on separate and consecutive days on the EPM, open field test (OF), and marble burying test (MBT) before exposure to ABA conditions. Blood samples were taken before behavioral testing and after recovery from the ABA paradigm to screen for proinflammatory cytokines. All experimental procedures were approved by the Monash Animal Resource Platform Ethics Committee (project ID:15171) and are detailed in Figure 1. A singly housed male rat was present in each experimental room to synchronize the estrous cycles of the female rats (known as the Whitten effect; Cora et al., 2015).

2.2 | Behavioral testing

Behavioral tests were recorded with an overhead camera connected to a computer and analyzed with TopScan Lite tracking software using center of mass (V 2.0; CleverSys) and manually scored by an observer blinded to experimental condition.

2.2.1 | Anxiety-like behavior and general locomotor activity

The EPM consisted of an elevated 4-arm platform made of gray Perspex (70 cm long × 10 cm wide × 90 cm high) with two closed (40-cm high walls) and two open arms. Rats were placed in the center

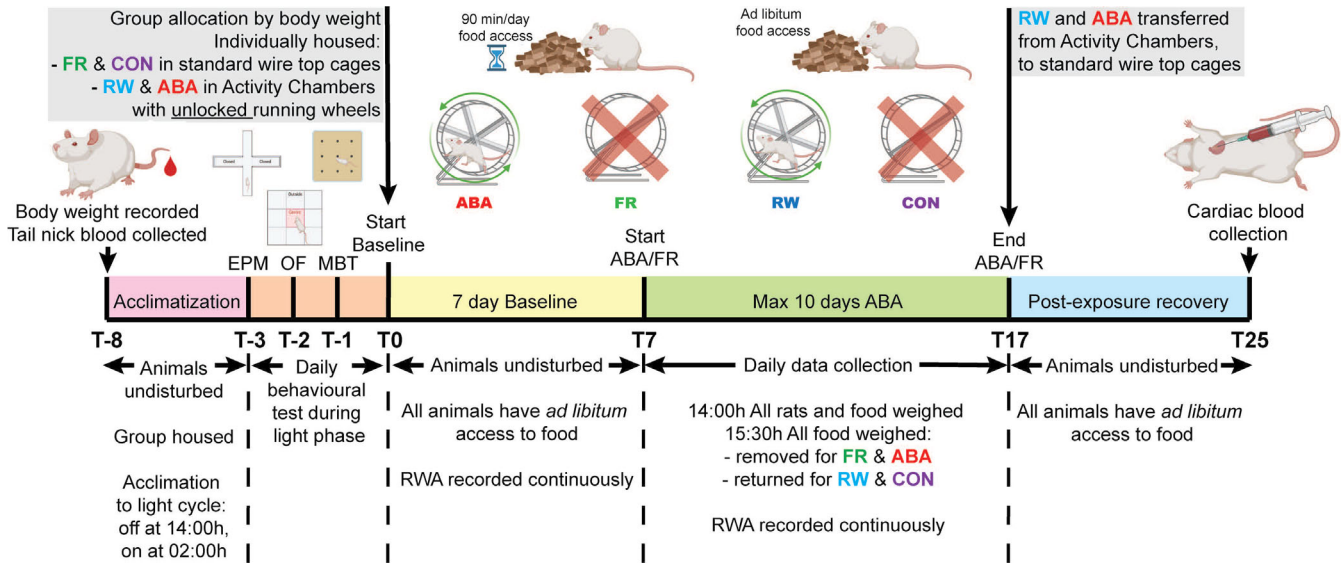

FIGURE 1 Experimental timeline. Pre-exposure blood samples were taken 8 days prior to the baseline testing period and rats were acclimated to the light cycle before maze-based behavioral tests on separate and consecutive days. The baseline testing period involved ad libitum food access and running activity was recorded for groups with access to wheels. Exposure to ABA conditions lasted for a maximum of 10 days, and all animals recovered with ad libitum access to food and no running wheels for 7 days postexposure, followed by terminal blood collection

platform (10 × 10 cm) facing an open arm and the proportion of time spent in the closed arms relative to the open arms in each 10-min trial, was used as the primary measure of anxiety-like behavior (File et al., 2004). The third (23 cm) of the open arms most distal to the central platform, considered the most "risky", was analyzed separately. Frequency of head-dipping and rearing behavior was also recorded to assess aspects of exploration. The OF test consisted of a deep open topped box (60 × 60 × 55 cm deep) in which distance traveled in each 10-min trial was used as the primary measure of locomotor activity and the proportion of time spent in the aversive center zone (middle square of a 3 × 3 grid; 20 × 20 cm) was used as a secondary measure of anxiety-related behavior, although it is shown to be less sensitive to the effects of anxiolytic drugs (Prut & Belzung, 2003). Assessment of vertical exploration was obtained during this test by recording the frequency of rearing.

2.3 | Compulsive behavior

The MBT consisted of the same OF box described above with a 5-cm deep layer of sawdust bedding on which nine marbles (blue glass; 8 mm diameter) were placed in an even grid pattern. The "classical" measure of compulsiveness in the MBT is number of marbles buried at the end of the test duration (in this case 10 min), where a greater number indicates increased compulsive tendencies (Albelda & Joel, 2012; Thomas et al., 2009). However, this measure does not account for the likelihood that rats will bury and unbury marbles throughout the test, and produces a confounding ceiling effect (Lazic, 2015); therefore, the number of interactions with marbles (including burying and unburying) was used as the primary measure of compulsive behavior in this study.

2.4 | ABA and experimental controls

Four experimental groups were used to examine the influence of behavioral and immune factors on susceptibility to ABA, and rats were allocated to groups to ensure an even spread of starting body weights. Animals either underwent exposure to the ABA paradigm (ABA; $n = 20$), had ad libitum access to food paired with access to a running wheel (RW; $n = 8$), had time-limited food restriction with no wheel access (FR; $n = 8$), or ad libitum food access and no running wheel (control CON; $n = 8$; see Figure 1). Twenty rats were allocated to the ABA condition to allow for the emergence of susceptible and resistant populations with adequate numbers in each. ABA and RW groups were individually housed in transparent activity wheel and living chambers (Lafayette Instruments; model 80859) whereas FR and control groups were singly housed in standard wire topped polypropylene cages. All groups were housed in a temperature (22–24°C) and humidity (30%–50%) controlled room under a reversed 12-h light/dark cycle (lights off at 1400 h). Food access for ABA and FR groups was restricted to 90 min at the onset of the dark phase (1400–15:30 h). Food restriction lasted for a maximum of 10 days and ABA conditions persisted until rats reached ≤80% baseline body weight or for a maximum of 10 days, whichever occurred first, at which point ad libitum access to food was reinstated until rats recovered to >100% of their baseline body weight ("post-exposure recovery"). Body weight and food intake were recorded daily between 1330 and 1400 h over the 10-day experimental period, with 90-min food intake also recorded at 1530 h.

2.5 | Assessment of running wheel activity (RWA)

Each running wheel (35.56 cm diameter) was equipped with an Activity Wheel Counter (Lafayette Instruments), which was connected by USB interface to a computer running Activity Wheel Software (Lafayette Instruments). RWA was recorded in 10-min intervals and RWA in the hour before feeding 1300–1400 h was used to assess food anticipatory activity (FAA). To control for individual differences in the propensity to run, FAA was calculated as a proportion of total daily RWA and food-restriction evoked hyperactivity was calculated for individual rats as the change in running from baseline to ABA (ABA-Baseline).

2.6 | Plasma collection and cytokine analysis

Blood was collected from tail tip at 6 weeks of age, 5 days prior to the commencement of behavioral testing. Approximately, 350 μl of whole blood was collected into EDTA-coated tubes (Microvette; Brand), which were centrifuged for 10-min (8000 rpm, 4°C) within 30 min of collection and plasma separated and stored at −80°C until use. Following exposure to ABA conditions, and including 7 days of ad libitum food access and body weight recovery to >100% baseline to ensure that effects of susceptibility to ABA were not confounded by the acute effects of starvation, blood was collected via cardiac puncture. A custom rat multianalyte LEGENDplex bead-based immunoassay kit was used to examine cytokine concentrations in plasma samples (LEGENDplex; BioLegend) that targeted six cytokines concurrently (IL-6, IL-10, IL-4, IL-1β, TNF-α, RANTES). These analytes were selected based on their previously reported elevation in human AN patients and/or ABA mice. Plasma samples were screened with the LEGENDplex assay kit as per manufacturer's instructions, and the readout measurement acquired using a Fortessa X-20 flow cytometer (Becton Dickinson [BD]). Data were analyzed using LEGENDplex Data Analysis Software (v8.0; BioLegend).

2.7 | Statistical analyses

Except otherwise noted all statistical analysis was performed in GraphPad Prism 8.0 (GraphPad Software). Significance for all tests was set at $p < .05$. A variety of statistical analyses were used determined by the type of data and number of groups: One- and two-way analysis of variance, followed by post hoc multiple comparisons (Tukey's or with a Bonferroni correction) when applicable; independent samples t test; Pearson's correlation; and linear regression. Details of each

individual analysis and complete statistical results can be found in Supplementary Information S1.

2.8 | Exclusions

One rat exposed to ABA showed an abnormal body weight loss trajectory and one rat exposed to FR was unable to maintain body weight >80% of baseline; data collected from both these animals were excluded from all analyses. Concentrations of IL-10, TNF-α, and IL-1β were not detected at levels high enough to be reliable using the LEGENDplex assay so these analytes were excluded from all analyses. A number of RANTES, IL-4 or IL-6 samples also contained concentrations that were below the threshold of detection of the assay and were excluded from analyses. Final sample sizes for all experimental groups and analytes are detailed in Appendix S1.

3 | RESULTS

3.1 | Effects of individual components of the ABA paradigm on weight maintenance and feeding

Of rats exposed to ABA conditions (Figure 2a), 9/19 (47%) were resistant to body weight loss, defined by an ability to maintain body weight above 80% of baseline for the entire 10-day experimental period (Figure 2b). While weight loss was negatively correlated with food intake for resistant animals, in line with all three control groups (all *ps* < .0001), there was no such association for rats susceptible to ABA ($p = .1610$; Figure 2c). Moreover, weight loss trajectories for resistant rats plateaued similarly to rats without access to a running wheel (FR; Figure 2d), whereas susceptible rats lost more body weight on average per day than both resistant and FR rats (both *ps* < .0001; Figure 2e). Access to a running wheel alone did not produce significant weight loss (RW, $p = .1446$; Figure 2f) but did increase food intake compared to control rats ($p = .0003$; Figure 2g,i) presumably to compensate for increased energy expenditure from wheel running. Much like the pattern of body weight loss, resistant rats ate a similar amount of food to FR rats ($p = .9982$; Figure 2g,h) whereas susceptible rats ate significantly less than both other food restricted groups (both *ps* < .0001; Figure 2g,h).

3.2 | RWA predicts susceptibility to weight loss under ABA conditions

Consistent with our previous findings (Milton et al., 2018), running activity *specifically* in the 2 days prior to food restriction predicted susceptibility to pathological weight loss in ABA, and food restriction-evoked hyperactivity was blunted in animals resistant to weight loss

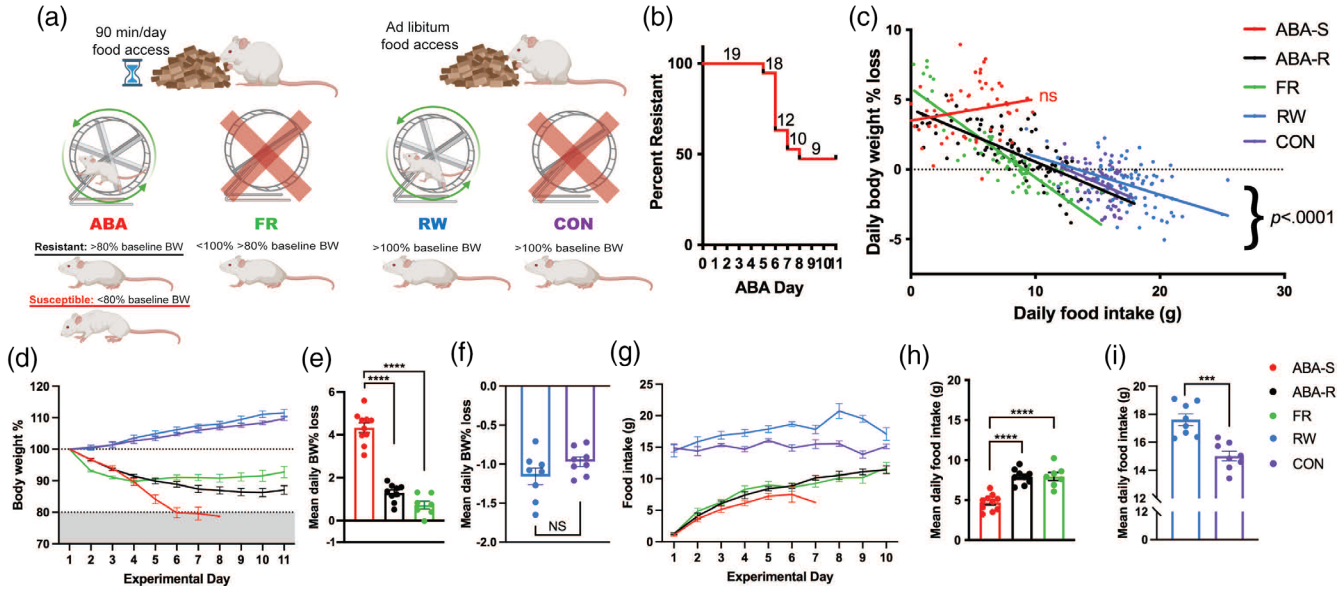

FIGURE 2 Body weight and food intake following exposure to individual or combined components of the ABA paradigm. (a) Five experimental groups: susceptible to ABA (ABA-S); resistant to ABA (ABA-R); sedentary rats with food restriction (FR); ad libitum fed rats with access to running wheels (RW); ad libitum fed sedentary controls (CON). (b) Plot of animals remaining in the ABA paradigm over time: 9/19 (47%) rats were resistant to ABA. (c) Linear regression with line of best fit and each individual daily data point for all animals (full results in Table S1). Daily food intake versus daily body weight %loss: ABA-R ($r = .7248$), FR ($r = .8712$), RW ($r = .4486$), and CON ($r = .4434$; all *ps* < .0001); ABA-S ($p = .1610$). Daily body weight percentage (d) and daily food intake (g) over the experimental period; group mean ± *SEM*. Mean daily body weight %loss (e, f) and food intake (h, i); individual animals (circles) and group mean ± *SEM*. (e, h) One-way ANOVA (results in Table S1) followed by Tukey's post hoc multiple comparisons. (f, l) Independent samples *t* test (results in Table S1). (e) ABA-S > ABA-R and RW (both *ps* < .0001). (f) RW versus CON ($p = .1446$). (h) ABA-S < ABA-R and RW (both *ps* < .0001). (i) RW > CON ($p = .0003$). $p > .05$, ***$p < .001$, ****$p < .0001$. NS, not significant $p > .05$

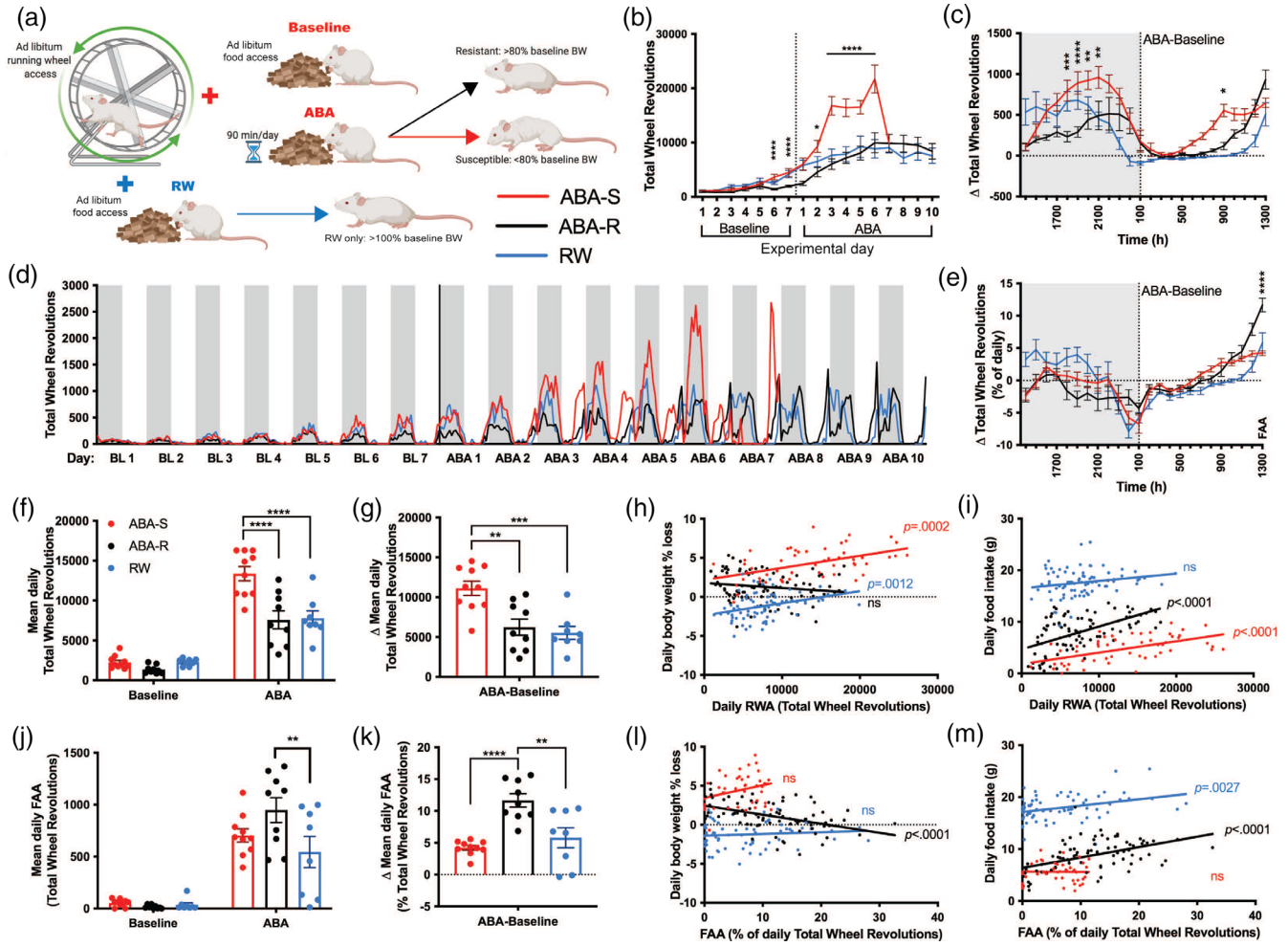

FIGURE 3 RWA for rats with wheel access either maintained on ad libitum feeding (RW) or combined with food restriction (ABA). (a) Three experimental groups: susceptible to ABA (ABA-S); resistant to ABA (ABA-R); ad libitum fed rats with access to running wheels (RW). Group mean ± *SEM* of daily running wheel activity (RWA) across the entirety of the experiment (b), change in mean hourly RWA across the dark (gray) and light (white) phases from baseline to ABA periods as absolute wheel revolutions (c), and as a proportion of total daily RWA (e). (d) Hourly RWA across dark (gray) and light (white) phases for the entirety of the experiment; group mean without error shown for clarity. (b, c, e) Two-way RM ANOVA (results in Table S2) followed by post hoc multiple comparisons with a Bonferroni correction were performed only on the ABA groups, RW is graphed solely for visual comparison. (b) Baseline: ABA-S > ABA-R ($p = .0101$); ABA-S > ABA-R Days 6 and 7 (both *ps* < .0001). ABA: ABA-S > ABA-R ($p < .0001$); ABA-S > ABA-R Day 2 ($p = .014$) and Days 3–6 (all *ps* < .0001). (c) ABA-S > ABA-R 1800–2100 h (all *ps* < .0063) and 0900 h ($p = .0156$). (e) ABA-R > ABA-S 1300 h ($p < .0001$). Mean daily RWA (f) and food anticipatory activity (FAA) (j) during baseline and ABA phases, and change in mean daily RWA (g) and proportional FAA (k) from baseline to ABA; individual animals (circles) and group mean ± *SEM*. (f, j) Two-way RM ANOVA (results in Table S2) followed by post hoc multiple comparisons, Tukey's for between groups or with a Bonferroni correction for between phase. (g and k) One-way ANOVA (results in Table S2) followed by Tukey's post hoc multiple comparisons. (h, i, l, m) Linear regression with line of best fit and each individual daily data point for all animals (full results in Table S2). (f) ABA: ABA-S > ABA-R and RW (both *ps* < .0001). (g) ABA-S > ABA-R ($p = .0022$) and RW ($p = .0008$). (h) Daily RWA versus daily body weight %loss: ABA-S ($r = .4970$, $p = .0002$) and RW ($r = .3553$, $p = .0012$); ABA-R ($p = .1476$). (i) Daily RWA versus daily food intake: ABA-S ($r = .5574$, $p < .0001$) and ABA-R ($r = .5508$, $p < .0001$); RW ($p = .0774$). (j) ABA: ABA-R > RW ($p = .0028$). (k) ABA-R > ABA-S ($p < .0001$) and RW ($p = .0019$). (l) Daily FAA versus daily body weight %loss: ABA-R ($r = .4569$, $p < .0001$); ABA-S ($p = .0666$) and RW ($p = .4237$). (m) Daily FAA versus daily food intake: ABA-R ($r = .4876$, $p < .0001$) and RW ($r = .3484$, $p = .0027$); ABA-S ($p = .9293$). *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. ns, not significant $p > .05$

(all *ps* < .0001; Figure 3b). Food restriction-evoked hyperactivity was especially pronounced in susceptible rats during dark phase running (all *ps* < .0063), but also occurred in the light phase ($p = .0156$; Figure 3c). This shift toward light phase running in susceptible animals was evident by the third day of ABA, with the light-phase activity peak constituting almost all running activity in susceptible rats by ABA Day 7 (Figure 3d). Resistant rats also increase light-phase running during ABA, but preferentially within the hour preceding food access ($p < .0001$; Figure 3e), known as food anticipatory activity (FAA; Mistlberger, 1994). On average, there were no differences between groups during baseline running; however, susceptible rats show marked hyperactivity during ABA compared to both other groups (both

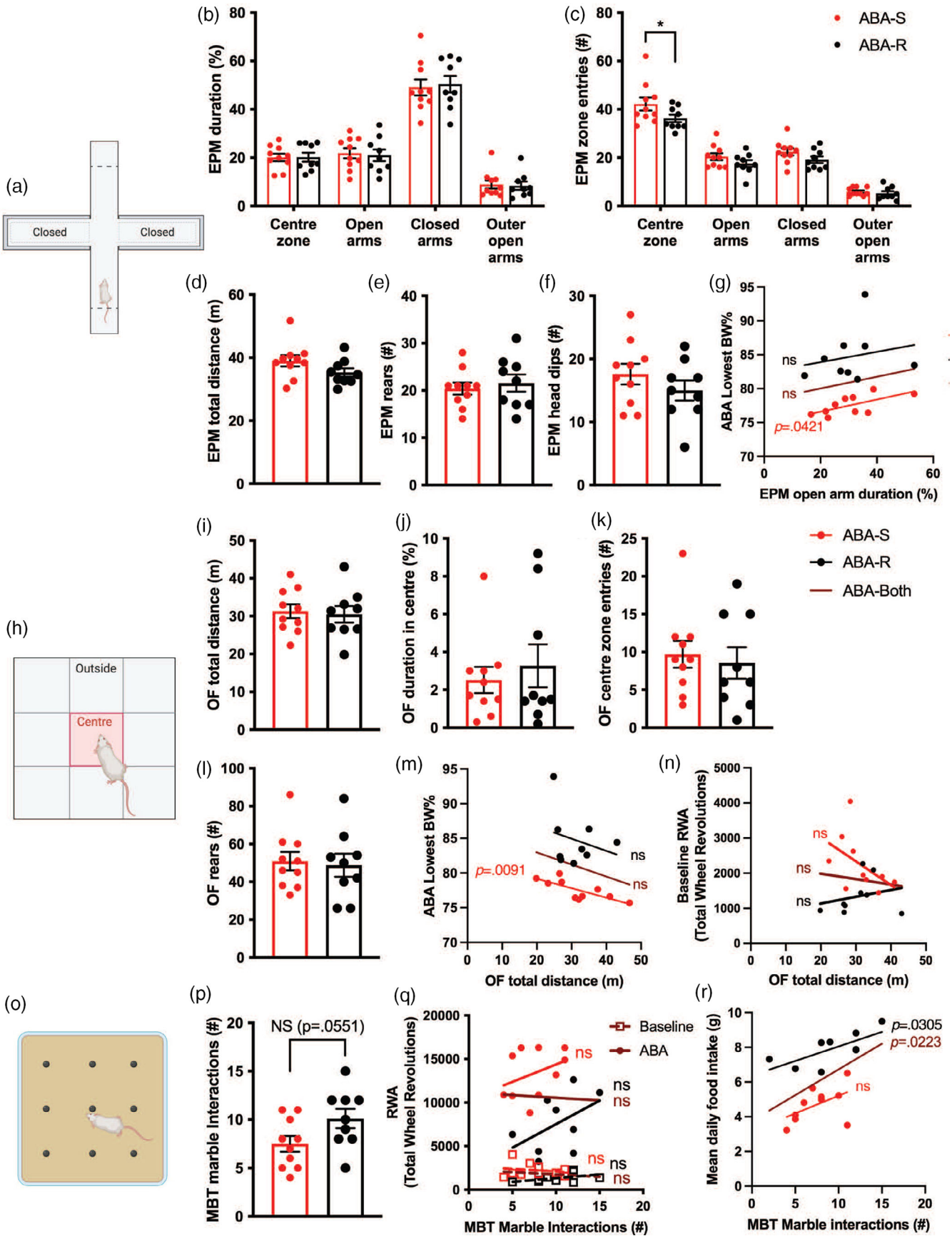

FIGURE 4 Legend on next page.

*p*s < .0001; Figure 3f). When we examined the change in running activity elicited by ABA in individual rats to account for baseline variability, hyperactivity remained specific for susceptible rats compared to resistant ($p = .0022$) and RW ($p = .0008$; Figure 3g) rats. Interestingly, daily running was positively correlated with body weight for RW ($p = .0012$) but not resistant ($p = .1476$; Figure 3h) rats and, conversely, associated with greater food intake for resistant rats ($p < .0001$) but not RW rats ($p = .0774$; Figure 3i) suggesting that while overall running did not differ between these groups, the relationship between running and other measures of energy balance did. While FAA, calculated as an average for each animal across both phases of the experiment (Baseline and ABA), was significantly elevated for resistant rats only over RW ($p = .0028$) but not susceptible ($p = .0736$) rats during ABA (Figure 3j), when differences in overall RWA between groups are taken into consideration, a significant elevation over both groups is revealed (see timepoint in Figure 3e; $p < .0001$). FAA during ABA was also significantly increased for resistant animals compared to both susceptible ($p < .0001$) and RW ($p = .0019$) groups when expressed as a change from baseline levels (Figure 3k), and was inversely associated with body weight loss only in resistant animals ($p < .0001$; Figure 3l). FAA is a motivated behavior that occurs in periods of food scarcity to increase the likelihood of finding food. It is therefore worth noting that while FAA was positively associated with food intake for resistant ($p < .0001$) and RW ($p = .0027$) animals, this association was absent in rats susceptible to ABA ($p = .9293$; Figure 3m).

3.3 | Increased anxiety-like behavior and hyperlocomotion were associated with a more severe ABA phenotype

The duration of time spent in different zones of the EPM (Figure 4a) did not differentiate between rats that went on to be susceptible or resistant to ABA ($p = .9979$; Figure 4b); however, rats that went on to be susceptible to ABA entered and exited zones more often than rats that went on to be resistant to ABA ($p = .0053$; Figure 4c), exemplified by more frequent crosses into the center zone ($p = .0355$), without an increase in locomotor activity ($p = .1215$; Figure 4d). The number of exploratory rears ($p = .6048$; Figure 4e) or head dips in the EPM ($p = .2709$; Figure 4f) did not differ between rats that went on to be susceptible or resistant to ABA; however, the primary measure of anxiety-like behavior on the EPM, duration spent in the open arms, was correlated with the extent of weight loss in ABA *for susceptible rats only* ($p = .0421$; Figure 4g). Similarly, while measures of locomotion, anxiety-like, or exploratory behavior in the OF (Figure 4h) did not predict susceptibility to ABA (distance traveled $p = .8629$, duration in center $p = .5724$, entries into center $p = .6799$, rearing $p = .7875$; Figure 4i–l), locomotor activity was correlated with the extent of weight loss in susceptible ($p = .0091$) but not resistant ($p = .4891$) rats (Figure 4m). Of note, given that RWA at baseline was predictive of ABA susceptibility but general locomotor activity was not, there was also no relationship between total distance traveled in the OF and baseline RWA (susceptible, $p = .1538$; resistant, $p = .5278$; Figure 4n). Moreover, while there was a trend toward increased interactions with marbles in the MBT (Figure 4o) for rats that went on to be resistant to ABA ($p = .0551$; Figure 4p), this measure was not correlated with RWA at baseline or during ABA (all *p*s > .1873; Figure 4q) but was positively associated with food intake for resistant ($p = .0305$) but not susceptible ($p = .1319$) rats (Figure 4r).

3.4 | Expression of proinflammatory cytokines IL-6 and IL-4 are associated with discrete ABA vulnerability factors

Analysis of blood samples taken before and after exposure to the various experimental conditions (Figure 5a) did not reveal significant group differences in the expression of RANTES (Figure 5b1–3), IL-4 (Figure 5c1–3), or IL-6 (Figure 5d1–3) However, baseline expression of IL-6 was positively associated with change in body weight for resistant but not susceptible rats ($p = .0305$ and $p = .4627$ respectively; Figure 5e), and the association between IL-6 and running activity was in opposite directions for susceptible and resistant rats, with increased IL-6 associated with higher running activity for

FIGURE 4 Pre-exposure behavioral test results and correlations with ABA outcomes. (a) Schematic of the EPM; outer open arms constituted the end one-third of each open arm. Bar graphs show group mean ± *SEM* with individual animals (circles) for animals that went on to be susceptible (ABA-S) or resistant (ABA-R) to ABA. (b, c) Two-way RM ANOVA (results in Table S3). (b) Duration (%) spent in each of the EPM zones did not differ significantly between groups. (c) Number of entries into each of the EPM zones. Center zone: ABA-S > ABA-R ($p = .0355$). (d–f, i–l, p) Independent samples *t* test (results in Table S3). Neither the total distance (d), number of exploratory rears (e), nor number of head dips (f) on the EPM differed between groups (all *p*s > .1215). (g, m, n, q, r) Linear regression with line of best fit and all individual animal data points; line of best fit for the combined ABA group is also shown (full results in Table S3). (g) EPM open arm duration versus ABA lowest body weight %: ABA-S ($r = .6495$, $p = .0421$); ABA-R ($p = .5801$) and combined ABA group (ABA-both; $p = .4248$). (h) Schematic of the OF. None of the OF outcome measures differed between groups (all *p*s > .5724): (i) total distance; (j) center duration (%); (k) center zone entries; (l) exploratory rears. (m) OF total distance versus ABA lowest body weight %: ABA-S ($r = .7706$, $p = .0091$); ABA-R ($p = .4891$) and combined ABA ($p = .2753$). (n) OF total distance versus mean daily baseline RWA: All *p*s > .1538. (o) Schematic of the MBT. (p) The number of interactions with marbles was not significantly different between groups ($p = .0551$). (q) MBT marble interactions versus mean daily RWA: All *p*s > .1873. (r) MBT marble interactions versus mean daily food intake: ABA-R ($r = .7147$, $p = .0305$) and combined ABA ($r = .5205$, $p = .0223$); ABA-S ($p = .1319$). *$p < .05$. ns, not significant $p > .05$

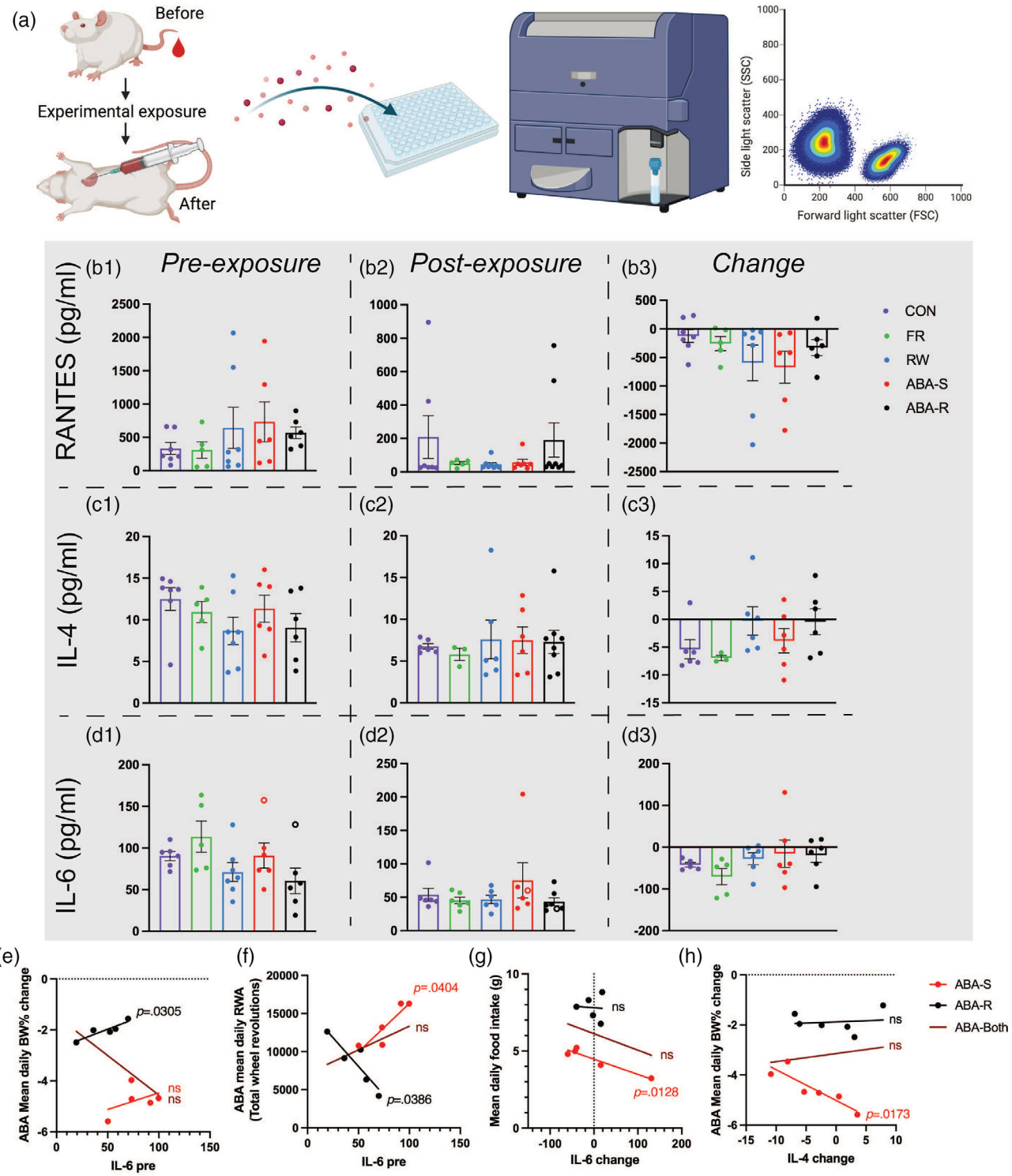

FIGURE 5 Concentration of proinflammatory cytokines before and after exposure to ABA conditions. (a) Schematic of blood collection and flow cytometry timeline. (b–d) Plasma cytokine concentration (pg/ml) collected from rats before experimental exposure (1), at the cessation of the experiment (2), and the change from before to after (3) for RANTES (b), IL-4 (c), and IL-6 (d). Welch's one-way ANOVAs revealed no significant differences between groups for any cytokine (all *p*s > .0581; results in Table S4). (e–g) Linear regression with line of best fit and all individual animal data points (full results in Table S4); Note that two outlying IL-6 pre-exposure values (1 from each ABA subgroup; open circles in (d)), and the corresponding change values, were excluded from linear regression analyses leaving $n = 5$ in each IL-6 group. (e) Pre-exposure IL-6 versus ABA mean daily body weight % change: ABA-R ($r = .9128$, $p = .0305$; ABA-S ($p = .4627$) and combined ABA (ABA-both; $p = .1396$). (f) Pre-exposure IL-6 versus ABA mean daily RWA: ABA-S ($r = .8947$, $p = .0404$) and ABA-R ($r = .8978$, $p = .0386$); combined ABA ($p = .2617$). (g) IL-6 change versus ABA mean daily food intake: ABA-S ($r = .9513$, $p = .0128$); ABA-R ($p = .9219$) and combined ABA ($p = .3963$). (h) IL-4 change versus ABA mean daily body weight % change: ABA-S ($r = .8907$, $p = .0173$); ABA-R ($p = .8211$) and combined ABA ($p = .7101$). ns, not significant $p > .05$

susceptible ($p = .0404$) but lower running activity for resistant ($p = .0386$; Figure 5f) rats. Moreover, the extent to which ABA elicited a change in IL-6 expression was associated with food intake only for susceptible ($p = .0128$) but not resistant ($p = .9219$) rats (Figure 5g). Finally, the change in IL-4 expression elicited by ABA was associated with increased weight loss for susceptible ($p = .0173$) but not resistant ($p = .8211$) rats (Figure 5h). See Figures S1–S5 for all correlational analyses.

4 | DISCUSSION

Mood-related comorbidities and immune dysregulation both contribute to increased risk and severity of AN in human patients. Here, we show that neither the behavioral correlates of anxiety, locomotion, and compulsivity nor circulating inflammatory markers that are associated with AN contribute to susceptibility to pathological weight loss in the ABA rat model. Contrary to expectation, classical measures of anxiety-like behavior or general locomotor activity did not differentiate between rats that went on to be susceptible or resistant to ABA. However, specific aspects of the ABA microstructure were differentially associated with behavioral phenotypes in susceptible versus resistant rats, providing new insight into why pathological weight loss occurs in some animals exposed to ABA conditions but not others. For example, increased anxiety-like behavior and hyperlocomotion were associated with a greater *extent* of weight loss in ABA for susceptible but not resistant rats, suggesting that these behavioral features contribute to increased weight loss in susceptible individuals. This is consistent with evidence that childhood or adolescent anxiety disorder symptoms are associated with lower BMI in individuals with AN (Dellava et al., 2010).

Moreover, rats that went on to be susceptible to weight loss in ABA demonstrated a significant increase in the number of crossings between zones on the EPM, which was driven by more frequent center crossings. Although cognitive behavior is not explicitly assayed with this test, the center of the EPM is considered to be a point of choice or decision-making, from which animals engage in high levels of risk assessment (Rodgers, 1997). Thus, if an animal is particularly indecisive about which arm to explore, the result is increased center platform activity (Nosek et al., 2008), which has been associated with inefficient responding in an operant conditioning task (Leonardo Rico et al., 2016). Deficits in decision making and cognitive flexibility are common to patients both currently ill with and weight-recovered from AN (Foldi et al., 2021; Sato et al., 2013; Steward et al., 2016; Tchanturia et al., 2012; Tenconi et al., 2016), and cognitive flexibility is impaired in rats after exposure to ABA (Allen et al., 2017). There is also evidence that cognitive dysfunction is a risk factor for general psychopathology in adolescents (Romer & Pizzagalli, 2021), and may therefore represent an endophenotype linking the comorbid development of mood disorders and AN. We have recently shown a neurobiological link between pathological weight loss in ABA and cognitive flexibility using touchscreen-based assays of operant responding (Milton et al., 2021). However, it should also be noted that *spatial cognition* has been shown to improve following weight restoration in ABA rats (Chowdhury et al., 2021), suggesting there are domain-based differences in the ways in which food restriction and excessive exercise influence cognitive outcomes. Future research should use translational assays of cognitive behavior, such as those based in touchscreen technology, *prior to* exposure to ABA conditions in order to interrogate this association.

This study also recapitulated our previous finding (Milton et al., 2018) that susceptibility to ABA could be predicted from baseline RWA in the 2 days prior to initiation of food restriction; however, the proportion of rats that were resistant to developing the ABA phenotype in this study was 47%—a marked increase from previous reports (Hurley, Collica, et al., 2021; Milton et al., 2018). It is likely that the additional handling required for maze-based behavioral testing is responsible for this increase, considering that repeated postnatal handling reduces vulnerability to ABA in adult rats (Carrera et al., 2006). It was also evident from this study that locomotor and running activity were not correlated for any group of animals that had access to wheels. This discrepancy highlights the fact that general locomotion and activity in the running wheel are distinct behaviors, likely underpinned by different neurochemical and motivational processes (Novak et al., 2012). Wheel running in ABA is also thought to be compulsive (Miletta et al., 2020); however, in this study, RWA was not associated with any measure on the MBT and in fact there was a trend toward increased "compulsive" marble burying for rats that went on to be *resistant* to ABA. This indicates that either compulsive behavior develops coincident with access to the wheel during ABA, rather than predisposes animals to pathological weight loss, or that the MBT is not a reliable measure of compulsivity in rats, but perhaps relates to investigative drive (de Brouwer et al., 2019). In support of the latter, marble burying was correlated with increased food intake for resistant but not susceptible rats, which may reflect an increased willingness to explore their environment and approach the food hopper more quickly when it is provided each day.

With respect to how circulating proinflammatory cytokines might predict susceptibility to ABA, there was no consistent group difference in expression of RANTES, IL-4 or IL-6 when sampled at the baseline, pre-exposure state. This is perhaps unsurprising considering changes in cytokine expression normally follow a "challenge"; however, it was interesting to see that IL-6 expression prior to exposure to ABA was associated with *increased* running activity for susceptible rats, but *decreased* running activity for resistant rats. It is clear that the intensity and duration of exercise are key factors in determining changes in inflammatory cytokine expression, and that exercise-mediated inflammation presents a different effect depending on the tissue type examined. For example, chronic or exhaustive exercise in rats both increases cytokine concentrations in skeletal muscle and reduces concentrations in adipose tissue (Gomez-Merino et al., 2007; Rosa Neto et al., 2009), whereas moderate exercise reduces expression of inflammatory cytokines in skeletal muscle (Lira et al., 2009) and in serum (Lin et al., 2021). Surprisingly, running activity appeared not to alter markers of inflammation in this study, with no significant changes in circulating cytokines elicited by either normal or excessive running behavior. Effects of caloric restriction on proinflammatory cytokine production is similarly tissue specific (Okita et al., 2012) but

both intermittent and chronic calorie restriction paradigms are shown to reduce serum IL-6 among other cytokines compared to ad libitum feeding (Dogan et al., 2017). There were no significant changes in cytokine expression elicited by food restriction in this study, but the change in IL-6 expression following exposure to ABA was associated with reduced food intake only for susceptible rats. It should be noted that postexposure blood samples were collected after body weight recovery to at least baseline levels, which also included a period of ad libitum food access for FR and ABA rats and no running wheels for RW and ABA rats. Considering the points above, and that elevated inflammatory markers seen in patients with AN have recently been shown to normalize with body weight recovery (Nilsson et al., 2020), it may be that a dysregulated inflammatory state is confined to the acute stage of anorexia in both humans and rats.

Despite some interesting hints that aspects of risk assessment and anxiety-related behavior could be used to predict susceptibility to pathological weight loss in the ABA model, this study did not identify a set of reliable behavioral or inflammatory features that predispose animals to ABA. It may be premature, however, to exclude such predisposing behaviors on the basis of the tests performed. These may lack the sensitivity or acuity to unveil underlying differences that will align with susceptibility to ABA, which may be revealed with more specific cognitive tests and/or "spike in" protocols for assessment of cytokine expression with larger sample sizes (Sullivan et al., 2000). Alternatively, there may be no single behavioral or immune-related measure that predicts susceptibility to ABA, but rather a raft of small differences that independently fail to predict susceptibility but, in concert, comprise a cumulative burden that results in pathological weight loss. This would fit with the difficulties in finding predictive markers for AN in humans and also explain the deficiencies of current treatments that often target only a subset of AN pathologies. It should be recognized that despite our intention to incorporate the most appropriate behavioral tests, other tests are available and predictive tests may be developed and utilized in the future, particularly with respect to cognitive mediators of susceptibility to ABA. It may also be the case that extensive handling and experimenter intervention should be avoided when examining factors that predispose to ABA, considering that repeated handling is known to alter susceptibility to weight loss (Carrera et al., 2006). It is now possible with novel technologies to examine behavior *without* experimenter intervention, for example using fully automated testing systems that rely on RFID technology (Caglayan et al., 2021; Zocher et al., 2020). Combining these automated systems with translationally relevant assays of cognition using touchscreens as described above offers a powerful tool for future investigations of the predictors of ABA.

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AUTHOR CONTRIBUTIONS

Laura Karina Milton: Data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Timothy Patton:** Formal analysis; investigation; methodology; writing – review and editing. **Meredith O'Keeffe:** Resources; supervision; writing – review and editing. **Brian John Oldfield:** Conceptualization; resources; supervision; writing – review and editing. **Claire Jennifer Foldi:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing.

CONFLICTS OF INTEREST

The authors declare no conflicts to interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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