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ORIGINAL ARTICLE

Ketogenic Food Ameliorates Activity-Based Anorexia of Adult Female Mice

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ABSTRACT

Objective: Genome-wide association studies implicate metabo-psychiatric origins for anorexia nervosa (AN). There are two case reports totaling six adult females who experienced complete remission of AN following a treatment comprised of ketogenic diet (targeting metabolism) with ketamine infusions (targeting psychiatric origins), but no study has determined the efficacy of ketogenic diet, alone. We addressed this gap in knowledge, with exploration of potential molecular mechanisms, using an animal model.

Method: Adult C57BL6 female mice underwent 2 or 3 cycles of activity-based anorexia (ABA1, ABA2, ABA3), an animal model of AN relapse, in which AN-like maladaptive behaviors of hyperactivity and voluntary food restriction are elicited when wheel access is combined with food restriction. ABA was categorized as severe, based on weight loss ≥ 20%, food restriction-evoked increase in wheel counts > 10,000/6 h, and crouching/grimace, and compared across two groups: (1) KG, fed ketogenic food continuously ($N = 25$); and (2) CON, fed standard diet ($N = 28$).

Results: 86% of CON versus none of the KG were crouching with grimace during ABA1. 93% of CON versus 11% of KG lost weight severely during ABA2 ($p < 0.001$, 8% difference of group mean weights). Severe hyperactivity was prevalent among CON (86%) and rare for KG (4%) during ABA2 ($p < 0.001$ on all food-restricted days). ABA up-regulated BDNF (brain-derived neurotrophic factor) in the hippocampus of both groups but ketone body, β-hydroxybutyrate, in urine was increased only among KG.

Discussion: Ketogenic diet may reduce severity of AN relapse through reduction of compulsive exercise, via mechanisms that are in addition to BDNF up-regulation and involve β-hydroxybutyrate.

1 | Introduction

Anorexia nervosa (AN) is a mental illness comprised of the key symptoms of compulsive voluntary food restriction (FR), severe weight loss, excessive exercise, and body dysmorphia (American Psychiatric Association 2013). AN's mortality rate is among the highest of all mental illnesses (Arcelus et al. 2011; Birmingham et al. 2005; Nielsen et al. 1998;

Abbreviations: 2HrFD, running during the food-accessible hours, 7–9 pm; ABA, activity-based anorexia; ABA1, first ABA, in late adolescence to young adulthood; ABA2, second ABA, in young adulthood; ABA3, third ABA, in adulthood; AgRP, agouti-related peptide; AN, anorexia nervosa; BMI, body mass index; FAA, food-anticipatory activity, 1–7 pm on food-restricted days; FR, food-restricted days; FR1, first day of food restriction during ABA; FR2, second day of food restriction during ABA; FR3, third day of food restriction during ABA; FR4, fourth day of food restriction during ABA; Kcal, kilocalories; KG, animal group that were fed ketogenic food in lieu of standard rat chow pellet starting from the wheel acclimation phase that preceded food restriction; KM, kilometers; MC4R, melanocortin receptor subtype 4; POMC, pro-opiomelanocortin peptide; WCT, wheel counts.

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Summary

- Anorexia nervosa is a mental illness with high incidences of relapse, especially in adulthood.
- In a mouse model of anorexia nervosa, ketogenic food protects against weight loss during food restriction, reduces hunger-evoked hyperactivity and reduces grimace/crouching.
- These findings suggest that ketogenic food and/or ketone bodies may be an effective treatment for patients diagnosed with anorexia nervosa by targeting metabolic origins, facilitating recovery and preventing relapse incidence.

Sullivan 1995), due to medical complications affecting multiple organs (Mehler and Brown 2015) and suicide (Smith, Zuromski, and Dodd 2018).

There is no accepted pharmacotherapy for the first onset or relapse of AN (Crow 2019). AN is often associated with anxiety that predates the diagnosis (Dellava et al. 2010; Kaye, Fudge, and Paulus 2009), but anxiolytics have been disappointingly ineffective (Steinglass et al. 2014). The atypical antipsychotic, olanzapine, reduces excessive exercise (Hillebrand et al. 2005), and increases weight, but is of limited benefit (Fornaro et al. 2023). The parent-enforced Maudsley Method (Lock et al. 2010) and cognitive behavioral therapies (Dalle Grave et al. 2014; Galsworthy-Francis and Allan 2014; Mulkens and Waller 2021) have been the most efficacious in redirecting patients' maladaptive thinking and behavior patterns that perpetuate the illness. Probability of relapse and persistence is higher among those that remain ill into adulthood, compared with those who recover while adolescent, and the Maudsley Method is less applicable to adults (Walsh 2013). These are reasons new treatments for AN, especially for relapsing adults, are needed.

Genome-wide association studies implicate metabo-psychiatric origins for AN (BMI-lowering alleles and genetic traits associated with obsessive-compulsive disorders, anxiety, schizophrenia, among others) (Duncan et al. 2017; Watson et al. 2019). Ketogenic (KG) diet holds promise as a treatment to target both origins of AN: normalizing metabolic pathways, as seen for diabetic patients, and also normalizing neuronal excitability, as seen for patients treated for epilepsy with KG diet (Paoli et al. 2023). Correspondingly, there is a case report of one adult (Scolnick et al. 2020) and a pilot study of five adults with AN (Calabrese et al. 2022), describing improvements in weight-maintenance and suppression of behavior they used to lose weight excessively after undergoing KG diet as a metabolic treatment, followed by sub-anesthetic ketamine, an FDA-approved anti-depressant. All six subjects were outpatients with restored weight prior to the study but with restrictive eating behavior before and no longer after the treatment. What remains unknown is whether KG diet, without ketamine, can ameliorate AN symptoms. We deemed this question to be important, because it is unclear when, if ever, ketamine will receive FDA-approval for treating AN and because ketamine can be addictive (Liu et al. 2016).

No study has yet revealed the scientific reasons behind KG diet's potentially ameliorative properties in treating AN. For patients battling AN, both hunger and satiety generate anxiety, and anxiety, regardless of its origin, propels the maladaptive behaviors patients use to lose weight excessively, that is, voluntary FR and excessive exercise (Kaye et al. 2004). One reason the above-described patients battling AN were able to reduce the maladaptive behaviors and enter long-term remission may be that KG diet reduces hunger (Paoli et al. 2015).

Although not listed as a core diagnostic feature in DSM-5 (diagnostic and statistical manual of mental disorders fifth Ed), in 40%–80% of the cases, AN is associated with excessive exercise that contributes to the severe weight loss (Beadle et al. 2015; Beumont et al. 1994; Carrera et al. 2012; Davis et al. 1997; Davis, Katzman, and Kirsh 1999; Hebebrand et al. 2003; Kron et al. 1978). Thus, "activity-based" anorexia (ABA) is a well-suited animal model for exploring treatments that reduce the AN-like maladaptive behavior of excessive exercise. For mice undergoing ABA, their wheel-running increases following FR and the extent of this increase correlates positively with both increases in FR-evoked anxiety-like behavior and with the extent of weight loss on days preceding hyperactivity, indicating that weight loss-evoked anxiety propels hyperactivity (Chowdhury et al. 2014; Wable et al. 2015). Thus, if a KG diet reduces weight loss and, thus, hunger experienced by mice during ABA, this would be predicted to reduce anxiety and with it, excessive exercise.

While reducing hunger, a KG diet could also paradoxically increase food intake through central pathways involving GABA, AMPK (5′-AMP-activated protein kinase), adiponectin (Paoli et al. 2015), and decrease anorexigenic POMC (pro-opiomelanocortin peptide) (Kinzig et al. 2005). For animals undergoing ABA without a KG diet, the orexigenic AgRP (agouti-related peptide) is increased (de Rijke et al. 2005). For ABA mice fed KG food, we may expect orexigenic behavior through increase of orexigenic AgRP concurrently with decrease of anorexigenic POMC. Accordingly, optogenetic stimulation of AgRP-containing neurons reduces mortality of ABA mice by improving coordination between physical activity and energy metabolism (Miletta et al. 2020). Moreover, mortality of male ABA rats fed a high fat diet is reduced (Barboriak and Wilson 1972). In this study, we used female ABA mice to assess how effectively KG food may reduce ABA vulnerability, measured as: (1) better weight retention; (2) reduction of excessive exercise; and (3) increased caloric intake.

ABA combines wheel acclimation with restricted food access (Routtenberg and Kuznesof 1967). In this environment, heightened exercise is induced by weight loss, yet underweight animals also voluntarily food-restrict by choosing to run, instead of eating, even during the limited hours of food access. As seen among patients with AN, many ABA animals exhibit anxiety-like behavior (Wable et al. 2015). Unlike mice that are food-restricted without wheels, mice undergoing ABA will die unless removed from the ABA-inducing environment before weight is reduced to >80% of pre-FR baseline (Chowdhury et al. 2013). Heightened exercise is readily inducible in mice (80% of adolescents (Chowdhury et al. 2013) and adults (Aoki and Santiago 2022)), probably because it is an innate foraging-like behavior (Gutierrez 2013). Even though foraging is adaptive in the wild, continuous running on a wheel is not adaptive for animals in captivity. When rechallenged with restricted food access (ABA2), adults will, again, lose weight precipitously due to hyperactivity and voluntary FR (Aoki

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and Santiago 2022). Thus, ABA captures five major symptoms of AN: excessive exercise, severe weight loss, voluntary FR, relapse and heightened anxiety. One core symptom not captured by the ABA model is body dysmorphia.

Some mice reduce wheel running when rechallenged with the ABA-inducing environment: they retain body weight better and survive. This behavioral change is accompanied by synaptic plasticity in the hippocampus and prefrontal cortex reflecting GABAergic suppression of excitatory neurons that is greater than the level seen among mice without ABA induction (Aoki et al. 2014; Chen et al. 2017, 2016; Chowdhury et al. 2014, 2013; Chowdhury, Fenton, and Aoki 2021). A major question remaining unanswered is how the ABA experience evokes this form of synaptic plasticity that is adaptive against relapses within a cage. Exercise (Cotman, Berchtold, and Christie 2007) and FR (Elesawy et al. 2021) enhance BDNF (brain-derived neurotrophic factor) synthesis and synaptic plasticity in hippocampus through generation of ketone, β-hydroxybutyrate, an endogenous histone deacetylase inhibitor that favors *Bdnf* gene expression (Sleiman et al. 2016). BDNF is required for growth and survival of neurons throughout the central nervous system and its importance is underlined by psychiatric (including depression) and neurodegenerative conditions that ensue when BDNF level declines (Esvald et al. 2023). BDNF and ketone body levels have never been assessed in ABA. We hypothesized that BDNF may become augmented more among ABA mice fed KG food than those fed standard diet, promoting greater synaptic plasticity and greater suppression of maladaptive behaviors. To begin testing this hypothesis, we addressed the following questions: (1) whether KG food ameliorates the maladaptive behaviors associated with ABA during the first onset (ABA1) and/or when rechallenged (ABA2, ABA3), and if so, whether this amelioration is associated with (2) greater synthesis of BDNF; and (3) greater synthesis of ketone bodies.

2 | Method

Animals: Animals were from 191, born at the animal facility of New York University, distributed across 10 cohorts. All were of C57BL6 background, with some from nonphenotypic

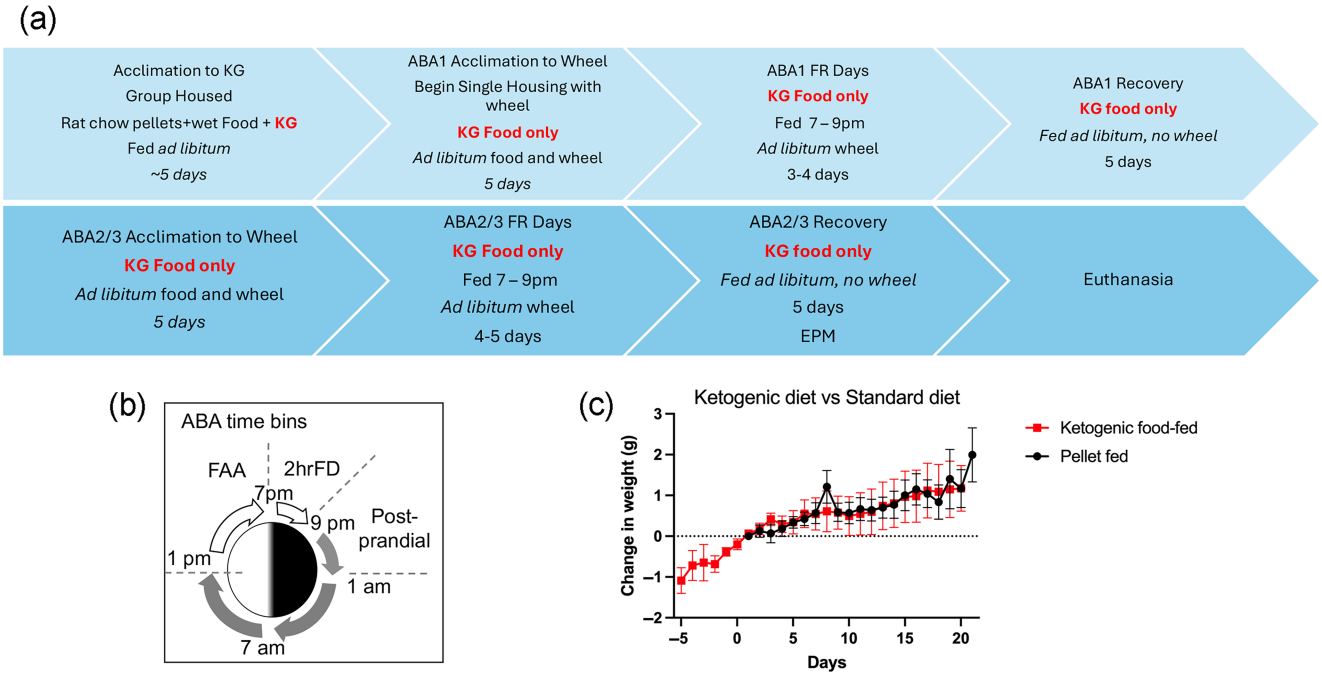


FIGURE 1 | The ABA schedule with ketogenic food. (a) Timeline of the multiple cycles of ABA and feeding schedule of ketogenic (KG) food. In brief, each cycle of ABA contained three phases: (1) Acclimation Phase to the wheel, during which time baseline wheel running, food consumption, and body weights (referred to as "weights" from here on) were assessed for each animal, by taking measurements once per day at around 1 pm. Low-profile wireless wheels (Med Associates, ENV-044) were fitted within each animal's cage. Acclimation lasted for 5 days; (2) FR Phase that began at 1 pm of FR1, in the presence of a wheel, by removing food from the cage. FR was imposed for three to four consecutive days during ABA1 (referred to as FR1, FR2, FR3, FR4) and for four to five consecutive days during ABA2 and ABA3. During FR days, food was available in unlimited amounts but only during the initial dark hours, from 7 to 9 pm ("2hrFD", Figure 1b); (3) Recovery Phase, with *ad libitum* food access and no wheel, spanning 5 days. After these three phases, animals were reacclimated to the wheel with *ad libitum* food access for 5 days (Phase 1), readying them for the FR Phase of ABA2, followed by the Recovery Phase of ABA2, then of the Acclimation Phase of ABA3, etc. Thus, 10 days of *ad libitum* food environment was interposed between the FR Phases of ABA2 and ABA3. All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by IACUC of New York University (A3317-01). (b) 24-h schedule on FR days of ABA. FAA = food anticipatory activity hours. (c) A pilot study was conducted to assess whether ketogenic food causes weight loss or gain and whether young adult mice can thrive on ketogenic food, alone. Weight changes of young adult mice fed standard rat chow pellet ($N = 9$, 3 of them males, black lines and symbols) versus ketogenic food ($N = 16$, 5 of them males, red lines and symbols) are shown. Day 0 is the day that pellet-fed animals switched to eating ketogenic food, only. Results indicate that young adults on either diet are still gaining weight. No group difference in weight gain was detected over the 3-week period. Values depict group mean ± SEM.

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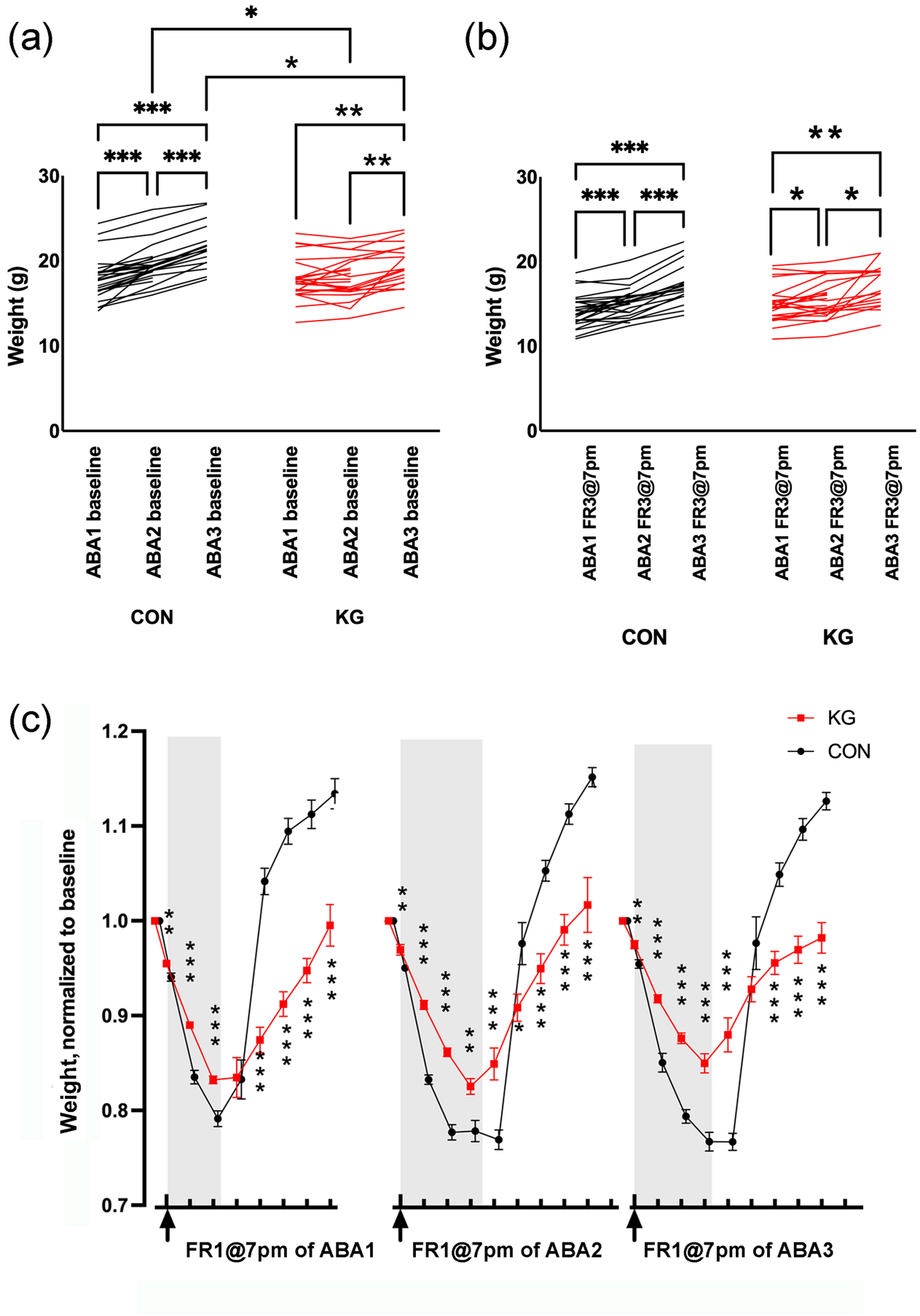


FIGURE 2 | Legend on next page.

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Cre-only or loxp-only knock-in stocks of Jackson Laboratory. We verified no difference in FR-evoked hyperactivity, weight or food consumption across these stocks (Goodwin-Groen, Dong, and Aoki 2023). Cohort effects were minimal (Supporting Information), and no data was excluded. Except for a small pilot study, which examined the impact of KG food on both males and females (Figure 1c), this study presents results from female mice, only ($N = 53$), to match the demographics of AN, which is 4–10 times more prevalent among females than males (Aoki and Santiago 2022; Smink, van Hoeken, and Hoek 2013).

2.1 | The ABA Procedure

Figure 1a shows the timeline of multiple cycles of ABA (ABA1, ABA2, ABA3). Please see Figure 1 legend and a previous publication (Aoki 2020) for details.

2.2 | The KG Food Treatment

The timeline of the KG food treatment is also shown in Figure 1a. Animals of 7 cohorts ($N = 25$) were allocated to the KG group. Of these, 3 cohorts ($N = 12$) were in the same cohort as of the control group of animals (CON) group, described below. While group-housed, KG were introduced to KG food (Bio-Serve Cat #F3666, 7.24 kcal/g, with 93.4% of the total kcal from fat, 1.8% from carbohydrate, 4.7% from protein) along with standard rat chow pellets (3.02 kcal/g, 13.12% kcal from fat, 24.5% from protein, 62.4% from carbohydrates) and a wooden block to mitigate teeth overgrowth. Simultaneous with introduction to the wheel, that is, 5 days prior to FR, animals began to be housed singly and food became only KG. In a pilot study, we verified that animals could sustain normal body weight while fed KG food, only, for multiple weeks (Figure 1c). On FR days, KG food was available only from 7 to 9 pm. At 9 pm, the cup containing KG food was replaced with Hydrogel water cups (Clear H_2O Hydrogel #70-01-5022, 0 Kcal/g). Assuming that ketosis is reached within 2 weeks after being fed KG food, only, under sedentary conditions (Benjamin et al. 2017; Kim and Rho 2008), this schedule was expected to induce partial ketosis during ABA1 and yield peak ketosis by the acclimation phase preceding ABA2.

The CON were distributed across 7 cohorts ($N = 28$), with 3 cohorts ($N = 15$) reared synchronously with KG. CON were fed standard pellets plus wet food (Clear H_2O DietGel 76A, 1 kcal/g, 18% kcal from fat, 17% from protein, 65% from carbohydrates) and their diet became hydrogel water cups, only, from 9 to 7 pm on FR days. Thirteen CON (3 cohorts) received intraperitoneal saline injections (10 mL/kg) once per day during re-acclimation and on FR days of ABA2. Since statistical analyses indicated no difference in their behaviors, compared to the 15 other CON without IP saline, the two CON sub-groups were combined for comparison with KG.

Euthanasia: Animals were euthanized using urethane (34%, 10 cc/kg IP) as the anesthetic, then either perfused transcardially with aldehydes for brain tissue preservation or decapitated, for measuring BDNF levels. Urine and feces were collected prior to anesthesia induction, frozen immediately using dry ice, then stored at −80°C.

Western blot BDNF analysis of the hippocampus: The hippocampus from a subset of brains of the KG and CON group that underwent ABA were analyzed for BDNF levels, as described previously (Sleiman et al. 2016), during the dark hours of ABA2. We also sampled the hippocampus from sedentary animals fed KG food ($N = 6$) or standard pellets ($N = 6$). We used anti-human BDNF primary antibody (Icosagen Cat#327-100, 1:1000), fluorophore-conjugated Odyssey IRDye-800 CW goat anti-mouse IgG (LI-COR Biosciences, 926-322-10 lot#030418-25, 1:10,000) as the secondary antibody, co-migration with human recombinant BDNF (Icosagen P-105-100) as the specificity control in Western blots, and REVERT Total Protein Stain (VWR 926-11016) to quantify protein loading/transfer per lane, after using the BCA protein assay kit (Pierce Cat#23225) to assess hippocampal lysates' protein concentration.

β-hydroxybutyrate (ketone body) levels in urine samples were measured by calorimetric assay (Cayman Chem Cat#700190) to confirm that KG food induced ketosis and to test whether ABA or FR, only ($N = 4$ fed KG food; $N = 7$ fed standard pellet), or exercise, only (EX, N = 7 fed KG food; N = 7 fed standard pellet), or neither (sedentary, $N = 9$ fed KG diet; $N = 10$ fed standard pellet) induced ketosis in the absence of KG food. Urine was collected just before euthanasia.

FIGURE 2 | Ketogenic food improves normalized weight retention during FR days of ABA. (a, b) Each line represents weights, in grams (not-normalized), of an animal of the CON (Black, $N = 28$) and KG (red, $N = 25$) groups, just prior to the start of FR days (baseline, panel a) and at 7 pm of the third day of FR, just prior to feeding (FR3@7 pm, panel b), compared across 3 cycles of ABA. Panel (a) shows that CON gained baseline weight consistently across ABA cycles, while KG gained baseline weight from ABA2 to 3 but not from ABA1 to 2. Panel (b) shows that, as expected, both groups lost weight by FR3 of each cycle. There was no group difference in their weights on FR3 of any ABA cycle, even though baseline weights were significantly less for KG than CON during ABA2 and 3. Here and in other panels of Figures 2, 3 and 4, *indicates $p < 0.05$; **indicates $p < 0.01$; ***indicates $p \leq 0.001$. Here and in other panels of Figures 2, 3 and 4, p values were calculated by a two-way ANOVA, with time and diet as the two factors, and with Tukey's correction for multiple comparisons. A mixed model was used, because repeated measures two-way ANOVA could not be performed always, due to some missing values, as animals exited FR when their weights became lower than 80% and or they became immobile with grimace. Exact p values of comparisons within groups are listed in Table 1. Exact p values of comparisons across groups are listed in Table 2. (c) Weights at 7 pm on FR days were normalized to the average of two values used to calculate baseline weight—that is, weight at 1 day prior to FR and at 1 pm on FR1, just prior to food removal. Weights were normalized to facilitate comparisons of weight changes across groups. The graph shows KG and CON's group mean ± SEM of normalized weights (y-axis) against days (x-axis) at 7 pm on FR days (shaded gray) and at 1 pm on 5 recovery days. CON's normalized weights were significantly less than KG's on all FR days of ABA1, ABA2, and ABA3 and were significantly greater than KG's on most recovery days. Exact p values for KG-versus-CON comparisons of normalized weights are listed in Table 2.

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TABLE 1 | Effects of diet over time across three ABA cycles: within-group comparisons.

Weights, not normalized			
Tukey's multiple comparisons test	**Mean diff.**	**95% CI of diff.**	**Adjusted *p* value**
CON			
ABA2 Baseline versus ABA1 Baseline	1.80	1.172 to 2.435	< 0.001
ABA2 FR3@7 versus ABA1 FR3@7	1.14	0.5082 to 1.772	< 0.001
ABA3 Baseline versus ABA2 Baseline	2.07	1.626 to 2.509	< 0.001
ABA3 FR3@7 versus ABA2 FR3@7	2.03	1.390 to 2.667	< 0.001
ABA3 Baseline versus ABA1 Baseline	3.87	3.111 to 4.632	< 0.001
ABA3 FR3@7 versus ABA1 FR3@7	3.17	2.498 to 3.839	< 0.001
KG			
ABA2 Baseline versus ABA1 Baseline	0.18	−0.5541 to 0.9097	0.973
ABA2 FR3@7 versus ABA1 FR3@7	0.69	0.0027 to 1.375	0.049
ABA3 Baseline versus ABA2 Baseline	1.39	0.4050 to 2.383	0.004
ABA3 FR3@7 versus ABA2 FR3@7	1.53	0.0881 to 2.971	0.034
ABA3 Baseline versus ABA1 Baseline	1.57	0.4050 to 2.383	0.007
ABA3 FR3@7 versus ABA1 FR3@7	2.22	0.6804 to 3.757	0.003
Caloric intake, not normalized			
Tukey's multiple comparisons test	**Mean diff.**	**95.00% CI of diff.**	**Adjusted *p* value**
CON			
ABA2 Baseline versus ABA1 Baseline	−1.55	−5.859 to 2.764	0.874
ABA2 FR3 versus ABA1 FR3	0.94	−1.330 to 3.202	0.779
ABA3 Baseline versus ABA2 Baseline	0.42	−4.926 to 5.774	> 0.999
ABA3 FR3 versus ABA2 FR3	0.64	−0.770 to 2.042	0.700
ABA3 Baseline versus ABA1 Baseline	−1.12	−2.605 to 0.3573	0.201
ABA3 FR3 versus ABA1 FR3	1.572	−0.248 to 3.392	0.113
KG			
ABA2 Baseline versus ABA1 Baseline	1.78	−1.671 to 5.227	0.6
ABA2 FR3 versus ABA1 FR3	−0.58	−1.887 to 0.7303	0.743
ABA3 Baseline versus ABA2 Baseline	−0.58	−5.159 to 3.998	0.998
ABA3 FR3 versus ABA2 FR3	1.82	0.6647 to 2.966	0.001
ABA3 Baseline versus ABA1 Baseline	1.20	−5.303 to 7.697	0.990
ABA3 FR3 versus ABA1 FR3	1.237	−0.2065 to 2.681	0.118

Note: Upper table: changes in weights over time across ABA1, 2, and 3 were followed for CON ($N = 28$) and KG ($N = 25$). CON increased their baseline weights significantly from ABA1 to 2 to 3. Correspondingly, their weights that reach minimum values at 7 pm, just before feeding, on FR3, were significantly increased from ABA1 to 2 to 3. However, the extent of weight retention improvement across the three ABA cycles at 7 pm were less than their baseline weight increases, indicating exacerbation of weight loss due to FR as the ABA cycles recurred. KG did not increase baseline weights from ABA1 to 2 but their minimal weights at 7 pm on FR3 increased significantly from ABA1 to 2 to 3. Comparisons across ABA cycles indicate that KG improved weight retentions at 7 pm as the ABA cycle recurred, more than their baseline weight gains across the same ABA cycles, reflecting gain of resilience as ABA cycles recurred. Lower table: changes in food consumption over time across ABA1, 2, and 3 were followed for the same CON and KG animals. The only significant change was on FR3 of ABA3 versus FR3 of ABA2 of KG, indicating gain of resilience as ABA cycles recurred for KG but not for CON. For both tables, *p* values are shown for repeated measure two-way ANOVA, with Tukey's corrections for multiple comparisons. Because some values were missing, these data were analyzed by fitting the mixed model. For these non-normalized weight data, diet × time interaction was significant ($p < 0.001$, $F(5,214) = 18.91$), the main effect of diet was not significant ($p = 0.508$, $F(1,51) = 0.4437$), and the main effect of time was significant ($p < 0.001$, $F(1.857,79.50) = 273$). For these non-normalized food consumption data, diet × time interaction was not significant ($p = 0.284$, $F(5,206) = 1.258$) while the main effects of diet and time were both significant ($p < 0.001$ for both, $F(1,51) = 16.36$ for diet, $F(3.081,127.0) = 69$ for time).

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TABLE 2 | KG versus CON Comparisons of weights and caloric intake during ABA.

	Mean diff (KG)–(CON)	95% CI of diff	Adjusted *p* value
Weights, not normalized			
ABA1 Baseline	0.09	−1.438 to 1.265	0.898
ABA1 FR3	0.81	−1.928 to 0.3071	0.151
ABA2 Baseline	−1.54	0.1840 to 2.895	0.027
ABA2 FR3	0.36	−1.511to 0.7929	0.533
ABA3 Baseline	−2.21	0.5003 to 3.926	0.013
ABA3FR3	0.14	−1.507 to 1.786	0.864
Weights, normalized to Baseline values			
ABA1 FR1	0.01	−0.02457 to −0.003869	0.008
FR2	0.05	−0.07104 to −0.03858	< 0.001
FR3	0.04	−0.05975 to −0.02198	< 0.001
Recovery 1	−0.00	−0.06167 to 0.05767	0.946
Recovery 2	−0.17	0.1280 to 0.2064	< 0.001
Recovery 3	−0.18	0.1438 to 0.2203	< 0.001
Recovery 4	−0.16	0.1252 to 0.2046	< 0.001
Recovery 5	−0.14	0.08409 to 0.1939	< 0.001
ABA2 FR1	0.02	−0.03314 to −0.005630	0.007
FR2	0.08	−0.09295 to −0.06507	< 0.001
FR3	0.08	−0.1034 to −0.06548	< 0.001
FR4	0.05	−0.07506 to −0.01860	0.002
Recovery 1	0.08	−0.1207 to −0.03915	< 0.001
Recovery 2	−0.07	0.01336 to 0.1217	0.016
Recovery 3	−0.10	0.06381 to 0.1430	< 0.001
Recovery 4	−0.12	0.08201 to 0.1623	< 0.001
Recovery 5	−0.13	0.07036 to 0.1990	< 0.001
ABA3 FR1	0.02	−0.03292 to −0.007803	0.002
FR2	0.07	−0.08965 to −0.04526	< 0.001
FR3	0.08	−0.1012 to −0.06367	< 0.001
FR4	0.08	−0.1115 to −0.05356	< 0.001
Recovery 1	0.11	−0.1545 to −0.07116	< 0.001
Recovery 2	−0.05	−0.01640 to 0.1133	0.134
Recovery 3	−0.09	0.05782 to 0.1288	< 0.001
Recovery 4	−0.13	0.08945 to 0.1646	< 0.001
Recovery 5	−0.14	0.1043 to 0.1841	< 0.001
Caloric intake, not normalized			
ABA1 Baseline	4.463	2.205 to 6.722	< 0.001
ABA1 FR3	2.122	0.8259 to 3.418	0.002
ABA2 Baseline	1.138	−2.440 to 4.717	0.525

(Continues)

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TABLE 2 | (Continued)

	Mean diff (KG)–(CON)	95% CI of diff	Adjusted *p* value
ABA2 FR3	3.636	2.078 to 5.194	<0.001
ABA3 Baseline	2.143	−1.232 to 5.518	0.203
ABA3FR3	2.457	1.316 to 3.598	<0.001
Caloric intake, normalized			
ABA1 FR1	0.007	−0.2466 to 0.2596	0.953
FR2	0.001	−0.2537 to 0.2563	0.992
FR3	0.021	−0.2485 to 0.2901	0.880
FR4	−0.022	−0.4729 to 0.4278	0.922
Recovery 1	0.351	0.08826 to 0.6130	0.009
Recovery 2	0.069	−0.1905 to 0.3281	0.603
Recovery 3	−0.040	−0.2995 to 0.2191	0.761
Recovery 4	−0.210	−0.4689 to 0.0497	0.113
Recovery 5	−0.444	−0.7062 to −0.1814	<0.001
ABA2 FR1	0.062	−0.2028 to 0.3265	0.647
FR2	0.121	−0.1433 to 0.3858	0.369
FR3	0.268	−0.0047 to 0.5397	0.054
FR4	0.148	−0.1532 to 0.4487	0.336
Recovery 1	1.078	0.7730 to 1.383	<0.001
Recovery 2	0.376	0.0713 to 0.6810	0.016
Recovery 3	0.384	0.0794 to 0.6891	0.014
Recovery 4	0.493	0.188 to 0.7977	0.002
Recovery 5	0.035	−0.2696 to 0.3401	0.820
ABA3 FR1	0.058	−0.2464 to 0.3633	0.707
FR2	0.102	−0.2199 to 0.4239	0.534
FR3	0.172	−0.1327 to 0.4770	0.268
FR4	0.184	−0.1441 to 0.5126	0.271
Recovery 1	1.533	1.163 to 1.903	<0.001
Recovery 2	0.653	0.2837 to 1.023	<0.001
Recovery 3	0.698	0.3630 to 1.032	<0.001
Recovery 4	0.524	0.1893 to 0.8583	0.002
Recovery 5	0.120	−0.2722 to 0.5188	0.549

Note: Comparisons of non-normalized baseline weights of CON ($N=28$) and KG ($N=25$) revealed no group difference at the start of FR of ABA1 but significantly less values for KG than CON at the start of FR of ABA2 and ABA3. Values of the two-way ANOVA of non-normalized weights appear in Table 1 legend. Because weights varied widely among animals, we normalized weights to facilitate comparisons across diet groups. Each animal's weight at 7 pm of each FR day and mid-day on recovery days 1 through 5 of each ABA cycle was normalized to its baseline weight at the start of FR of that cycle. Across all ABA cycles, KG were better than CON at retaining their weight during FR days. Conversely, CON restored their weights more quickly than KG. A two-way ANOVA was performed, with time (29 time points) and diet (2 types) as the two factors and with Tukey's corrections for multiple comparisons. Because some values were missing, these data were analyzed by fitting the mixed model. This analysis revealed a significant interaction of diet and time ($p<0.001$, $F(28,1079)=38.34$) and significant main effects of diet and time ($p<0.001$ for both, $F(1,51)=15.58$ for diet, $F(6.210,239.3)=144.5$ for time). The non-normalized caloric intake comparison between KG and CON showed that caloric intake was consistently greater for CON ($N=28$) than KG ($N=25$) preceding FR (i.e., Baseline). By the start of ABA2, baseline caloric intake of the two groups became similar. However, caloric intake was consistently less for KG than CON during FR across all 3 cycles of ABA. Values from the two-way ANOVA of non-normalized caloric intake appear in Table 1 legend. Because weights of animals varied greatly (range of ~10 g), we normalized caloric intake for each animal, to facilitate comparisons across groups. Baseline caloric intake of each animal was calculated as the average of the values of the 2 days prior to each ABA cycle. Baseline caloric inputs were used to calculate normalized caloric intake of FR days 1 through 4 and recovery days 1 through 5 of each ABA cycle. Normalized caloric intake was not significantly different across the two groups during any of the FR days but greater for CON than KG during many of the recovery days. Two-way ANOVA was performed, with time (29 days of ABA) and diet (2 types) as the two factors. *p* values were calculated from mixed-effect analysis with Tukey's corrections for multiple comparisons. This test revealed a significant diet × time interaction ($p<0.001$, $F(29,1016)=5.887$) and significant main effects of diet and time ($p<0.001$ for both, $F(29,1016)=54.19$ for time, $F(1,49)=19.29$ for diet).

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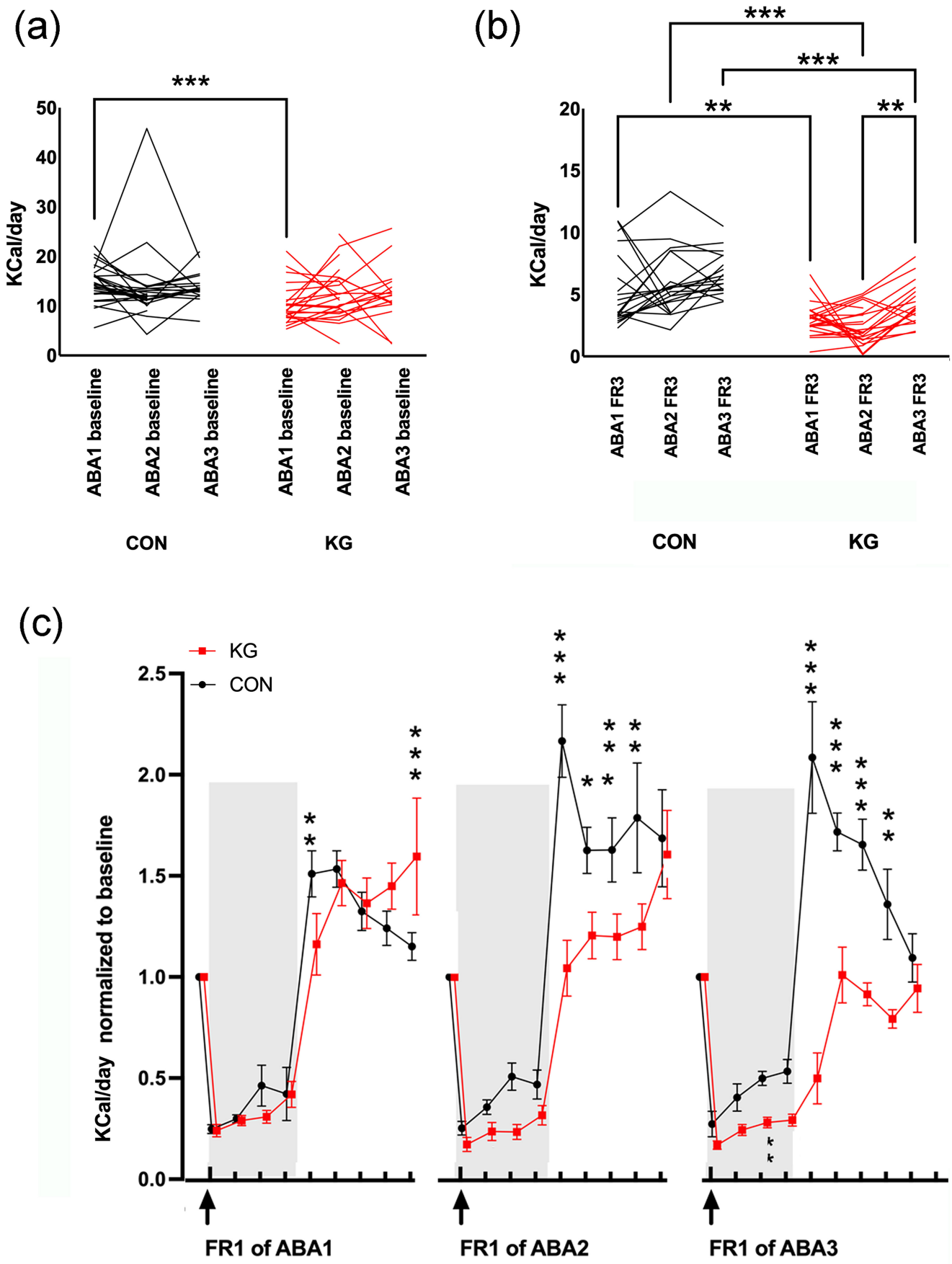


FIGURE 3 | Legend on next page.

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Statistical analyses: For testing the hypothesis that KG food alters weight retention, caloric intake or wheel exercise during ABA, we performed a two-way ANOVA, with repeated measures across all time points of ABA (i.e., time) as one factor and diet as the other, followed by Tukey's correction for multiple comparisons.

For comparing individuals' changes in weights and caloric intake across time and diet groups, we used averaged measurements from two time points for normalization: the moment before FR began at 1 pm and 1 day prior.

Pearson's correlation analyses were performed to examine relationships between behavioral measurements and BDNF levels in the hippocampus.

These tests and graphical representations were prepared, using Prism (GraphPad version 10.0.3).

3 | Results

3.1 | KG Food Minimizes Weight Loss During ABA Without Increasing Caloric Intake

Maintenance of animals on KG food, only, while acclimating to wheels for 5 days caused no significant difference in KG's group mean baseline weight, when FR of ABA1 began, compared to CON's (non-normalized data, Figure 2a, Table 2). By the start of FR of ABA2 and ABA3, CON gained more baseline weight, compared with their preceding cycles' baseline weights (Table 1, Figure 2a). By contrast, KG's baseline weights did not increase from ABA1 to ABA2 (Table 1, Figure 2a), thus becoming significantly although only slightly lower than CON's baseline weights for ABA2 (Figure 2a, Table 2).

Since their baseline weights within groups varied widely (~10 g, Figure 2a), we tracked each animal's percent changes in daily weights, relative to baseline values that preceded FR (i.e., normalized weights, Figure 2c, Table 2). During FR days, when food access became restricted to 2 h (from 7 to 9 pm, lights off), KG's normalized weights were significantly and consistently greater than CON's. This group difference was apparent at 7 pm of each FR day, coinciding with the end of 22 h of food deprivation (Figure 2c, Table 2, $p < 0.01$ for all FR days). Specifically, by the end of ABA1, CON's weights reduced to 70% of baseline but were at 83% of baseline for KG. By the end of ABA2, CON's weights were 78% of baseline and 82.5% of baseline for KG. By the end of ABA3, KG's normalized weights were again significantly higher: 82.5% of baseline for KG and 78% of baseline for CON.

Indications of discomfort as grimace (Whittaker, Liu, and Barker 2021) and crouching were obvious for 86% of CON, often requiring cessation of FR before the originally scheduled last day (FR4). Grimaces were less frequent for KG (0% at the end of ABA1, 33% at the end of ABA2, 21% at the end of ABA3) than CON, paralleling KG's less severe normalized weight loss when compared with CON, even though their non-normalized weights were not different from CON's (Figure 2b, Table 2). Upon return to *ad libitum* feeding, all weight losses due to FR were gained within 2 days for CON but took 4–5 days for KG (Figure 2c).

Are KG able to retain body weight during FR better than CON because they consume more calories during the hours of food availability? To address this question, we calculated animals' daily caloric intake. Caloric intake of KG was significantly less than CON's at entry time point for ABA1's FR, by which time KG had been fed KG food, only, for 5 days ($p < 0.001$, Figure 3a), but this group difference disappeared by the start of ABA2 and ABA3 (Table 2, Non-normalized and Figure 3a). By contrast, KG's food intake per day was significantly less than CON's on FR days of ABA1, ABA2, and ABA3 (Figure 3b). Since animals' weights varied widely and increased, as a group, from ABA1 to ABA2 to ABA3 (Figure 2a), we reasoned that animals' food intake would be affected by their body size (weight). Therefore, we also tracked percent daily changes in food consumption, after normalizing their food intake to baseline values before FR began. Comparisons of normalized food intake showed no group difference during any of the FR days of ABA1, ABA2, or ABA3 (Figure 3c, Table 2). Unlike the minor differences in normalized food consumption during FR, food consumptions during recovery days that followed FR were much greater for the CON than KG (Figure 3c, Table 2). This matches the temporal pattern of weight restoration during recovery, which was faster for CON than KG (Figure 2c). More importantly, these data indicate that KG's improved weight retention during FR of ABA1, 2, and 3 were not due to increased caloric intake.

KG run much less than CON: We next asked whether KG's improved weight retention during FR was linked to decreased wheel running. Among CON, food anticipatory activity (FAA) spanning the hours of 1–7 pm is a strong predictor of weight loss

FIGURE 3 | Ketogenic food does not increase caloric intake of animals undergoing ABA. (a, b): Each line represents caloric intake, in kcal (not normalized), of an animal of the CON (Black) and KG (red) groups, just prior to the start of FR days (baseline, panel a) and during the third day of FR, (panel b) compared across 3 cycles of ABA. Panel (a) shows that baseline caloric intake per day of KG was less than CON's, leading up to ABA1 (i.e., ABA1 ($p < 0.001$) but not significantly less for ABA2 or ABA3 baselines. This likely reflects waning of food neophobia (Coulthard, Aldridge, and Fox 2022). Panel (b) shows that, as expected, caloric intake reduced drastically when hours of food availability was reduced from *ad libitum* to 2 h per day on FR days. Non-normalized food intake on FR days was greater for CON than KG, here represented by intake on FR3. *p* values were calculated, after repeated measures two-way ANOVA, with time and diet as the two factors, and with Tukey's correction for multiple comparisons. Exact *p* values of within-group comparisons are listed in Table 1. Exact *p* values of across-group comparisons are listed in Table 2. (c) Food consumption (Kcal per day), normalized to baseline levels, latter of which were calculated as the average of caloric intake on the 2 days leading up to FR days. Asterisks reflect significant differences across groups. These values did not differ across diet groups during ABA1 but were significantly less for KG, compared with CON, during FR days of ABA2 and ABA3. During recovery, caloric intake of KG was consistently less than CON's for 3–4 days but surpassed CON's significantly by the 5th day of ABA1 and were indistinguishable from CON's for ABA2 and 3. Asterisks reflect *p* values from two-way ANOVA, as described in panels (a, b) but for normalized values. Exact *p* values are listed in Table 2.

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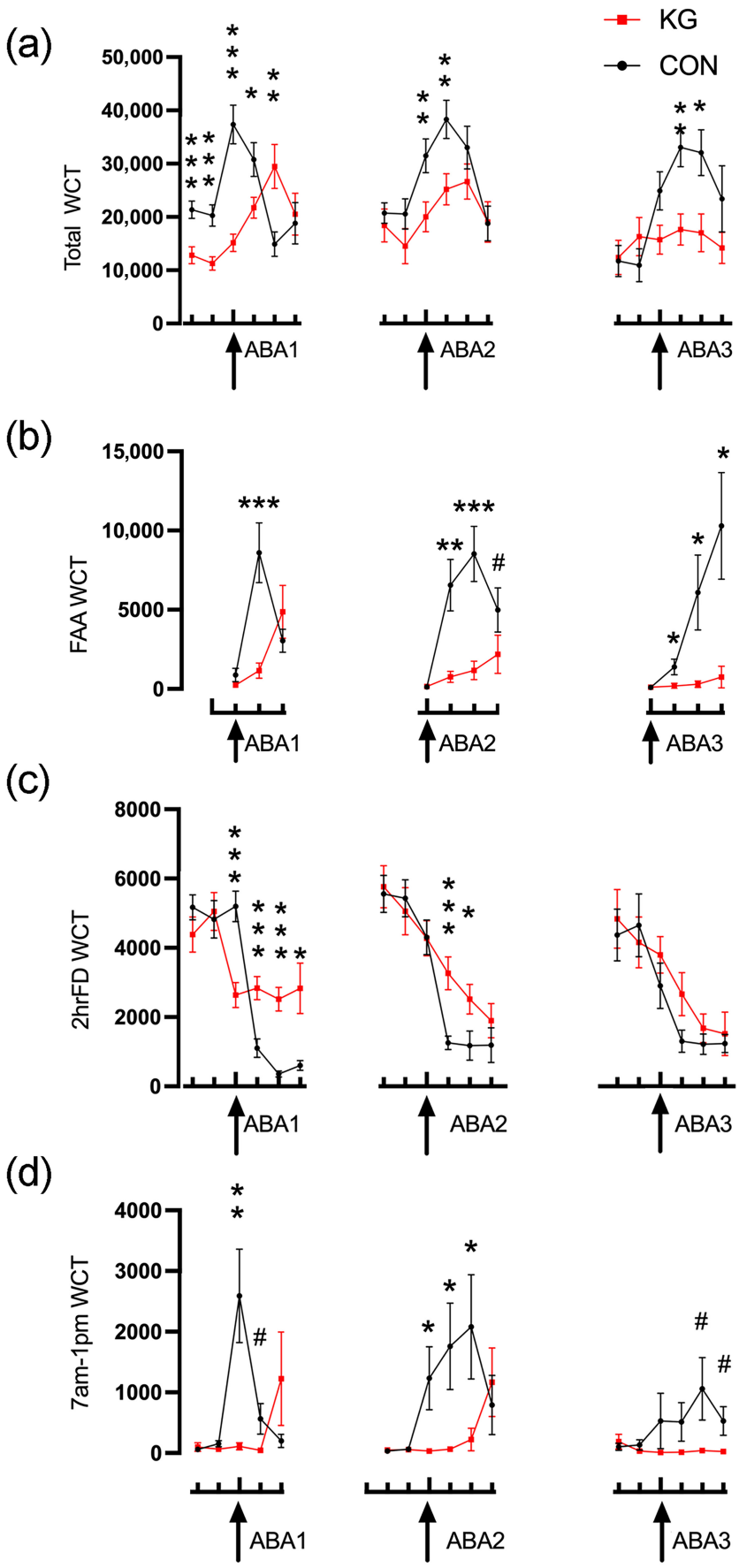


FIGURE 4 | Legend on next page.

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(Chowdhury et al. 2013). FAA wheel counts (WCT) of KG were significantly less than CON during FR2 of ABA1 ($p < 0.001$, Figure 4b and Table 3), with only 7 out of the 25 KG becoming severely hyperactive (> 10,000 WCT in 6 h), compared with 14 out of the 28 of CON. During ABA2, FAA of KG were even more dramatically reduced on FR2 and FR3, compared with CON, with only 1 among the 23 exhibiting severe FAA hyperactivity. By contrast, CON showed even greater FAA hyperactivity during ABA2, compared with ABA1 (17 out of 26 vulnerable). During ABA3, only 1 of the 17 of the KG exhibited severe hyperactivity, with the group average of KG being significantly lower than CON's during FR2, 3 and 4 of ABA3 ($p < 0.05$) (Figure 4b, Table 3). By contrast, CON's FAA did not diminish from ABA2 to ABA3, with 8 out of 18 continuing to be severely hyperactive.

The pattern of increase of total running per 24 h across the days of FR was like that of FAA and strikingly different across the two groups (Figure 4a, Table 3). During ABA1, CON reached peak levels of running 2 days earlier than KG and both groups decreased their total running by FR4 (Figure 4a). During ABA1 and ABA2, KG ran significantly less than CON on FR1 and FR2 ($p < 0.05$), and both groups diminished their wheel running by FR4. By ABA3, KG diminished their wheel running on FR2 and FR3 even more than during ABA2 ($p = 0.05$), but such a decline was not observed for CON ($p = 0.62$).

Animals' running during the light period preceding FAA, that is, 7–1 pm (Figure 4d, Table 3) was of similar chronological pattern to their FAA: KG's running was significantly less than CON's, and the FR-evoked increase in running was delayed by 2 days for ABA1 and ABA2 and not at all apparent for ABA3. KG's reduction, compared with CON, was the greatest for FR1 of ABA1 ($p = 0.003$) but remained significant on the first 3 days of FR of ABA2 ($p < 0.05$).

As weight loss accumulated from FR1 to FR2 to FR3 (Table 1), CON dramatically decreased wheel running during the 2 h of food availability (2hrFD, Figure 4c, Table 3), indicating that they were learning to choose to eat, rather than run, during the hours of food availability that they anticipated (Chowdhury et al. 2013). KG exhibited wheel running during the 2hrFD that were strikingly different from CON's, in that their extent of running did not decline as much from FR1 to FR2 to FR3, compared with CON ($p < 0.001$ for most days of ABA1 and ABA2). During ABA2, KG ran consistently and significantly more than CON on FR2 and FR3. Finally, by ABA3, the two groups' running pattern across the FR days converged. This chronological pattern parallels weight change across the FR days, which was also less severe for the KG, compared with CON (Figure 2a, Table 2) and their grimace, which was prevalent among CON but not for KG. These behaviors suggest that KG did not reduce wheel running during 2hrFD, because their hunger was less severe than CON's.

3.2 | Immunoblot Measurements of BDNF

What is the molecular mechanism underlying KG food's strong suppression of hyperactivity? Both exercise and KG diet raise the ketone body, β-hydroxybutyrate which, in turn, induces activity of key promoters of the *Bdnf* gene that enhances synaptic plasticity (Sleiman et al. 2016). To test whether BDNF was augmented more among KG than CON, we assayed BDNF levels in the hippocampus ($N = 6$ for each group), a brain region with highest levels of BDNF (Esvald et al. 2023; Sleiman et al. 2016). We also compared their values to those of sedentary animals fed the two types of diet ($N = 3$ for each diet group) (Figure 5a,b). Immunoreactivity of mature BDNF (mBDNF) was significantly different between hippocampi of ABA mice compared to sedentary mice ($p = 0.017$, $t = 2.662$, $df = 16$) (Figure 5b). However, we detected no consistent difference between KG and CON's levels of BDNF ($p = 0.50$, $t = 0.6917$, $df = 10$). Instead, CON and KG's levels jointly correlated strongly and positively with their Total WCT per day, summed across FR2 and FR3 of ABA2 (Pearson correlation $R = 0.72$, $p = 0.0088$, Figure 5c).

Ketone body increases due to KG food and especially following ABA: It was the diet-related difference in ABA due to different levels of ketone bodies? We presumed ketone bodies would be increased by KG diet but a possibility remained that ketone bodies were elevated in CON as well, since strenuous exercise, alone, and fasting, alone can lead to the generation of ketone bodies (Laeger, Metges, and Kuhla 2010; Newman and Verdin 2017; Sleiman et al. 2016). Urine levels of the ketone body, β-hydroxybutyrate, was significantly higher among KG, compared with CON ($p = 0.0064$, Figure 5d, "ABA"), and CON's level (pellet ABA) was no different from those of sedentary animals fed standard pellet diet ($p = 0.4555$, difference of the means ± SEM 0.455 ± 0.30 mM). By contrast, β-hydroxybutyrate in urine of sedentary animals fed KG diet

FIGURE 4 | Group comparisons of wheel counts (WCT) reveal significant reductions by the KG group during the hours without food availability and greater running during the hours of food availability. (a) Total WCT, representing the daily sum of wheel running across all 24 h: Total WCT of KG were consistently and significantly less than CON's across almost all FR days of ABA1, ABA2, and ABA3. The once exception was FR3 of ABA1. Here and in all other panels, arrows point to FR1, with tick marks on the *x*-axis to the left of arrows depicting days preceding FR. Overall, KG's hunger-evoked hyperactivity increase was delayed by 2 days during ABA1 and blunted on all FR days of ABA2 and ABA3. *N* values and methods of 2-way ANOVA are as described under Figure 2. (b) Food-anticipatory activity (FAA) WCT during 1–7 pm on FR days was consistently and significantly less for KG, compared with CON. The one exception was the last FR day of ABA1, when KG's FAA crossed over the values of CON. Overall, KG's FAA increase was delayed by a day during ABA1, relative to CON's, blunted during ABA2 and altogether nonexistent during ABA3. By contrast, CON's FAA was high throughout the three ABA cycles. FAA WCT on non-FR days are not shown, because they were nearly zero. (c) Wheel running during the 2 h of food availability (2hrFD) from 7 to 9 pm was one time bin during, which KG exhibited greater running than CON. These group differences disappeared by ABA3. (d) The light hours preceding FAA, namely 7–1 pm, is when non-ABA animals are dormant but ABA animals are active (Chowdhury et al. 2013). This pattern was observed for CON. For KG, there was a 2-to 3-day delay in the emergence of running during this period of ABA 1 and 2 and none during ABA3. # indicates $0.05 < p < 0.1$. Group comparisons were based on a two-way ANOVA, with time and diet as the two factors, followed by Tukey's corrections for multiple comparisons. Exact p-values are listed in Table 3.

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TABLE 3 | Comparison of CON versus KG wheel counts during ABA.

CON versus KG WCT comparisons	Mean diff (CON)–(KG)	95% CI of diff	Adjusted *p* value
FAA WCT			
ABA1 FR1	630.4	−293.2 to 1554	0.174
ABA1 FR2	7443	3459 to 11427	< 0.001
ABA1 FR3	−1831	−5533 to 1871	0.322
ABA2 FR1	−32.76	−233.1 to 167.6	0.740
ABA2 FR2	5782	2370 to 9194	0.002
ABA2 FR3	7358	3609 to 11107	< 0.001
ABA2FR4	2802	−916.3 to 65	0.136
ABA3 FR1	−9.111	−135.5 to 117.3	0.884
ABA3 FR2	1200	119.8 to 2280	0.031
ABA3 FR3	5790	786.6 to 10794	0.026
ABA3 FR4	9539	2224 to 16855	0.014
Total WCT			
ABA1 Pre2	8520	3955 to 13084	< 0.001
ABA1 Pre1	8989	4198 to 13781	< 0.001
ABA1 FR1	22203	14148 to 30259	< 0.001
ABA1 FR2	9042	1501 to 16583	0.020
ABA1 FR3	−14571	−24113 to −5028	0.004
ABA1 FR4	−1720	−12895 to 9456	0.757
ABA2 Pre2	2344	−5281 to 9970	0.528
ABA2 Pre1	6027	−2944 to 14997	0.179
ABA2 FR1	11457	2923 to 19991	0.010
ABA2 FR2	13117	3793 to 22441	0.007
ABA2 FR3	6404	−4059 to 16868	0.224
ABA2 FR4	−286.4	−10486 to 9913	0.955
ABA3 Pre2	−661.5	−9692 to 8369	0.880
ABA3 Pre1	−5373	−15219 to 4473	0.270
ABA3 FR1	9164	−38.81 to 18366	0.051
ABA3 FR2	15424	5962 to 24887	0.002
ABA3 FR3	15037	3705 to 26369	0.011
ABA3 FR4	9229	−5157 to 23.616	0.195
7–1 pm WCT			
ABA1 Pre2	−40.85	−193.1 to 111.4	0.5758
Pre1	85.75	−44.44 to 215.9	0.1914
FR1	2479	895.2 to 4063	0.0034
FR2	521.1	−3.281 to 1046	0.0514
FR3	−1027	−2633 to 578.1	0.1995

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TABLE 3 | (Continued)

CON versus KG WCT comparisons	Mean diff (CON)–(KG)	95% CI of diff	Adjusted *p* value
ABA2 Pre2	−18.79	−86.17 to 48.59	0.5678
Pre1	6.291	−67.38 to 79.96	0.8640
FR1	1197	126.9 to 2267	0.0298
FR2	1696	220.6 to 3171	0.0261
FR3	1857	41.46 to 3672	0.0454
FR4	−377.9	−1902 to 1147	0.6169
ABA3 Pre2	−82.73	−387.6 to 222.2	0.5534
Pre1	104.0	−91.60 to 299.5	0.2646
FR1	516.1	−472.0 to 1504	0.2795
FR2	498.8	−184.1 to 1182	0.1386
FR3	1020	−97.57 to 2137	0.0703
FR4	502.2	−54.89 to 1059	0.0705
2HrFD WCT			
ABA1 Pre2	785.9	−488.4 to 2060	0.217
Pre1	−223.6	−1792 to 1345	0.774
FR1	2558	1414 to 3702	< 0.001
FR2	−1735	−2600 to −871.1	< 0.001
FR3	−2163	−2888 to −1437	< 0.001
FR4	−2229	−3867 to −590.3	0.012
ABA2 Pre2	−208.6	−1850 to 1433	0.798
Pre1	372.6	−1386 to 2131	0.670
FR1	26.11	−1412 to 1465	0.971
FR2	−2006	−3062 to −950.8	< 0.001
FR3	−1341	−2549 to −132.0	0.030
FR4	−710.0	−2127 to 706.5	0.317
ABA3 Pre2	−465.5	−2879 to 1948	0.687
Pre1	493.7	−1923 to 2910	0.676
FR1	−895.2	−2600 to 809.8	0.293
FR2	−1360	−2798 to 79.18	0.063
FR3	−459.5	−1497 to 577.7	0.373
FR4	−280.2	−1698 to 1138	0.684

Note: A two-way ANOVA was performed with time and diet as the two factors, with Tukey's correction for multiple comparisons. For all time bins, there were significant effects of diet, time and diet × time interactions, with KG exhibiting less WCT than CON, except during 2hrFD. For FAA WCT, time × diet interaction was significant ($p < 0.001$, $F(10,419) = 5.032$) as were the main effects of diet and time ($p < 0.001$ for both, $F(1,51) = 36.50$ for diet, $F(5.231,219.2) = 5.834$ for time). For Total WCT, the time × diet interaction was significant ($p < 0.001$, $F(17,643) = 5.207$), diet's main effect was marginally significant ($p = 0.055$, $F(1,51) = 3.859$) and the main effect of time was highly significant ($p < 0.001$, $F(6.496,245.7) = 7.728$). For the 7–1 pm light hours WCT, interactions of diet and time was highly significant ($p < 0.0001$, $F(16,584) = 3.461$), the main effect of diet trended towards significance ($p = 0.05$, $F(1,51) = 3.960$) and the main effect of time effect was significant ($p < 0.05$, $F(5.141,187.6) = 2.853$). For the 2hrFD WCT, the interaction of diet and time was highly significant ($p < 0.0001$, $F(17,651) = 4.437$) and the main effect of diet was significant ($p = 0.025$, $F(1,51) = 5.343$) as was the main effect of time ($p < 0.001$, $F(6.744,258.3) = 24.37$). Unlike other hours of the day, KG exhibited higher WCTs during the 2 h of food availability. $N = 28$ for CON, $N = 25$ for KG.

were significantly increased, relative to the levels of pellet-fed sedentary animals ($p = 0.0002$, difference of the means ± SEM 1.032 ± 0.2233 mM, Figure 5d, "Sedentary"). KG-ABA animals' β-hydroxybutyrate levels were augmented due primarily to the condition of FR, revealed by comparing β-hydroxybutyrate levels of KG diet-fed sedentary animals (KG FR) with and without

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FR (compared with KG Sedentary, $p = 0.0413$, difference of the means ± SEM 4.906 ± 1.711 mM), and not due to exercising $p = 0.9995$, comparing β-hydroxybutyrate levels of KG food-fed animals on wheels for 21 days (KG EX) versus KG food-fed sedentary animals (KG Sedentary) (Figure 5d).

4 | Discussion

Delays in effective intervention of AN until adulthood are strong predictors of relapses that dominate mental health for years (Walsh 2013). With the hope of meeting the challenges of

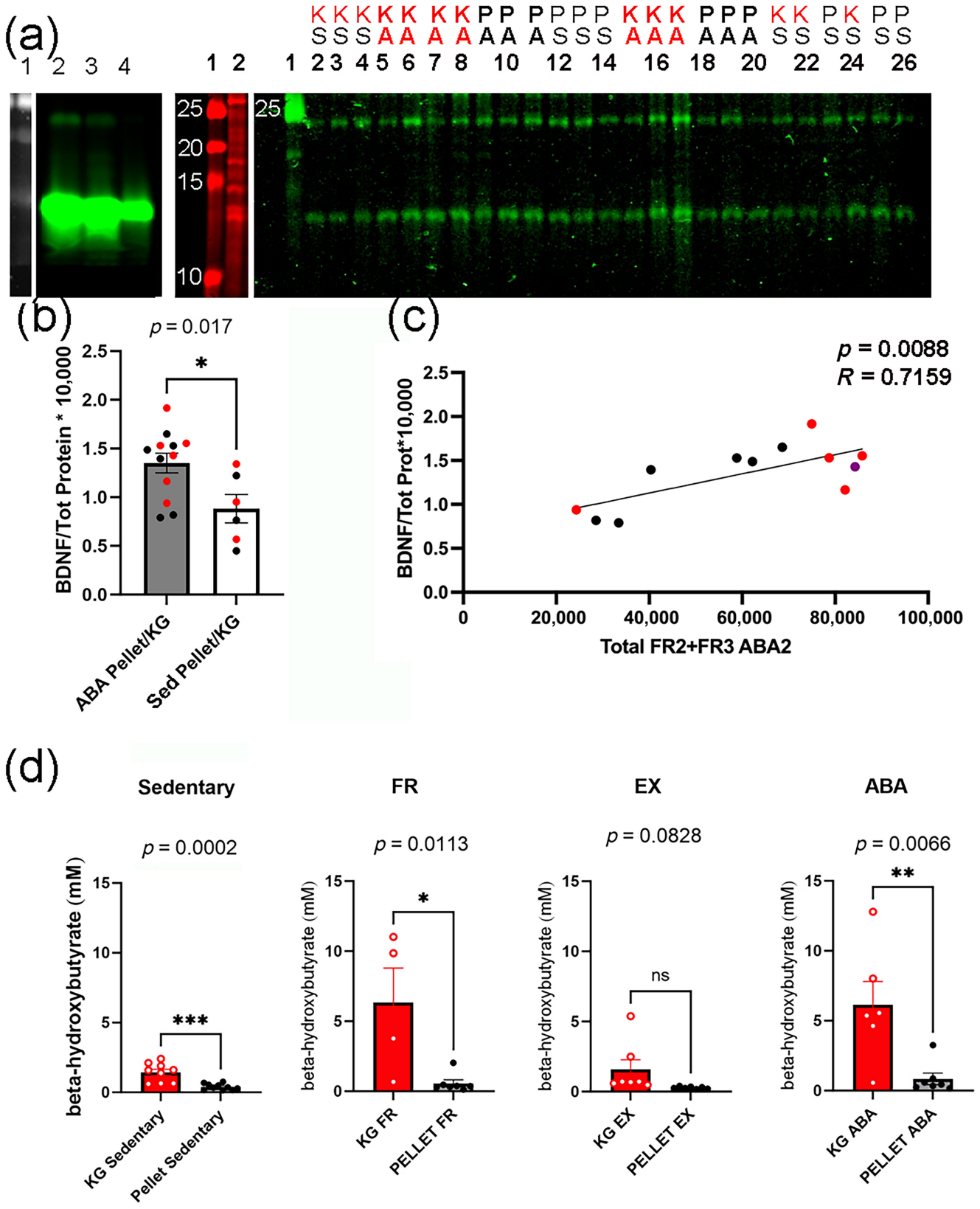


FIGURE 5 | Legend on next page.

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treating adults suffering from AN and AN relapse, this study explored the potential use of KG food to reduce anorexia-like maladaptive behaviors of adult mice.

As observed in past studies, control animals on the standard pellet diet (CON) experienced severe hunger that accumulated from the first to the last day of FR which, in turn, evoked severe hyperactivity during the hours without food and less hyperactivity during the hours with food. Wheel activity pattern of animals fed KG diet was the opposite, suggesting that KG experienced less severe hunger, thereby exhibiting less FR-evoked hyperactivity overall and during FAA, when animals were most severely underweight. KG's greater activity during the 2hrFD, relative to CON's, also is consistent with the idea that they experienced hunger less severely. These results indicate that FR using KG food, alone, is more effective than standard diet for reducing anorexia-like maladaptive behaviors of hyperactivity and distress (crouching and grimace) during the first onset of ABA as well as during relapses.

As for the mechanism by which KG food reduces the maladaptive behaviors, we first hypothesized that this was through an augmented synthesis of BDNF. However, this was found not to be. We verified that elevation of β-hydroxybutyrate is sustained by KG diet, even under sedentary conditions, and elevated much more by ABA, while remaining negligible without KG diet, even under the conditions of ABA. These results indicate that KG food is protective against ABA through mechanisms linked not only to BDNF synthesis but possibly through a sustained rise of ketone bodies. Since a KG diet has higher caloric density than standard diets, patients may be able to approach caloric intake necessary for weight maintenance more readily by consuming smaller volumes of KG food than of standard diet. This may be a goal more readily achievable, especially for patients struggling to curtail hyperactivity and relapse. KG's lag in weight restoration following FR, compared with CON, points to the obvious—namely that a well-balanced diet comprised of standard diet supplemented with KG food would be helpful for restoring health quickly.

The largest genome-wide association studies to date of AN points to significant genetic correlations with metabolic and lipid traits (Watson et al. 2019). Our findings align well with this conclusion, but also suggest that shift from carbohydrate to fat as energy source through KG diet can strongly curb anorexia-like maladaptive behaviors, even for individuals without metabolic and lipid traits, like our wildtype mice.

4.1 | Mechanism of Action of KG Food Treatment

Although the mechanism of the ameliorative effects of KG diet remains elusive, as it was 100 years ago in the treatment of childhood epilepsy, the number of molecular candidates is mounting, as are the diseases proposed to be targeted effectively by the diet (Field et al. 2022). ABA animals' WCT tallied across 24 h correlated significantly and positively with BDNF levels in the hippocampus, as observed earlier for exercising rats without FR (Cotman, Berchtold, and Christie 2007). Contrary to our expectation, BDNF levels were heightened equally for the two diet groups, compared to levels found in brains of sedentary animals, even though CON did not generate detectable levels of ketone bodies (Figure 5c). It appears that the great increase in exercise prompted by the ABA paradigm exceeds the ketone body-dependent regulatory mechanism that induces BDNF expression (Sleiman et al. 2016).

β-hydroxybutyrate, verified to be generated by KG diet among animals of our study, is more than a ketone body metabolite. It binds to and inhibits class I histone deacetylaces (Shimazu et al. 2013), causing up-regulation of many genes besides the *Bdnf*, including those involved in longevity pathways for neuroprotection and stress resistance (Newman and Verdin 2017). More studies are needed to understand what changes, other than BDNF, are induced by the rise of ketone bodies.

KG food may suppress FR-evoked hyperactivity through changes in hypothalamic brain regions that are sensitive to increased anorexigenic signal, cholecystokinin, and reduced orexigenic signal, ghrelin (Paoli et al. 2015), thereby reducing FR-evoked

FIGURE 5 | Immunoblot comparing BDNF in the hippocampus of ketogenic food fed (K) vs. standard pellet fed (P) ABA mice (A) vs. sedentary mice (S). (a) The white bands in the left-most lane 1 shows molecular weight markers for 25, 20, and 15 kDa. The immediately adjacent green fluorescent bands of lanes 2–4 show immunoblot of BDNF peptide (lane 1: 8 μg, heated to 95°C for 10 min; lane 2: 8 μg, heated to 95°C for 5 min; and lane 3: 8 μg, not heated). The red fluorescent bands in lanes 1 and 2 show molecular weight markers and protein bands from a lysate after staining with REVERT total protein stain and scanned at 800 nm. The green bands of lane 1 are molecular weight markers scanned at 700 nm. Lanes 2–26 show lysates from 25 different mice immunolabeled using the same anti-BDNF antibody. Lysates loaded in the lanes were as follows: Lanes 2–4, 21, 23, and 24 from ketogenic food-fed sedentary mice (KS); Lanes 5–8 and 15–17 from ketogenic food-fed ABA mice (KA); lanes 12–14, 23, 25, and 26 from sedentary mice fed standard pellet diet (PS); and lanes 9–11 and 18–19 from ABA mice fed standard pellet diet (PA). Fluorescence intensity of the 14 kDa immunoreactive band was measured for assessing BDNF levels in lysates, then normalized using total protein stain. (b) Unpaired *t* test showed significant difference in BDNF level of ABA-animals, compared with sedentary animals, regardless of the diet fed. BDNF level was normalized using total protein stain transferred to the immunoblot membrane. Each data point represents value from one animal. Red = ketogenic food-fed ABA, that is, KG; Black = pellet-fed ABA, that is, CON. "Sed" represents data of sedentary animals fed ketogenic diet or rat chow pellet and never exposed to a wheel or FR, after excluding 6 data points from those samples collected during light hours (lanes 2–4 and 12–14). All others were sampled during the evening hours. There was no difference between the two diet sub-groups within the ABA or Sed groups. (c) Pearson correlation analysis of ABA animals' wheel running during 24 h of FR2 plus of FR3 of ABA2, relative to BDNF shows significant correlation, indicating that those animals that ran more also expressed higher levels of BDNF. There was no difference in correlation across the two diet groups. (d) β-hydroxybutyrate levels (BHB) of urine from KG versus CON, compared with levels in animals that were sedentary ($N = 9$ for ketogenic, $N = 10$ for pellet), or were on wheels only (EX, $N = 7$ for both ketogenic and for pellet) or experienced FR only ($N = 4$ for KG, 7 for pellet). ABA significantly increased BHB of KG-ABA more than CON-ABA, and this augmentation was ascribable to the experience of FR more than exercise. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns = non-significant.

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excessive exercise of ABA mice. This is what we observed for KG (Figure 4, Table 3). Importantly, ketone bodies are reported to also increase food intake, even with reduced hunger (Paoli et al. 2015), but this is not what we observed.

5 | Conclusion, Limitations of the Current Study and Future Directions

Many questions remain unanswered. What duration of KG diet is maximally effective for curtailing maladaptive behaviors while also preventing potential negative effects, such as cellular senescence (Wei et al. 2024)? Is there a sex-difference in the efficacy of KG diet? Since many individuals battling AN dislike high fat (Foerde et al. 2015), can treatments of supplementing diet with ketone bodies bypass the need for KG diet? The potential benefit of co-administering KG diet with ketamine, as seen for patients battling AN (Calabrese et al. 2022) is also worthy of pursuit using this animal model (Aoki 2024).

Author Contributions

Yiru Dong: data curation, formal analysis, investigation, methodology, validation, visualization, writing – review and editing. **Yuki Lin:** formal analysis, investigation, visualization. **Latika Khatri:** data curation, formal analysis, investigation, methodology, supervision, validation. **Moses Chao:** conceptualization, data curation, investigation, project administration, resources, supervision, writing – review and editing. **Chiye Aoki:** conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be provided upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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