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# Three daily intraperitoneal injections of sub-anesthetic ketamine ameliorate activity-based anorexia vulnerability of adult female mice

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## Abstract

**Objective:** To identify ketamine's dosing schedule that ameliorates voluntary food restriction, hyperactivity and body weight loss of adult mice undergoing activity-based anorexia (ABA), an animal model of anorexia nervosa.

**Method:** Female and male C57BL6 mice underwent three cycles of ABA, starting from mid-adolescence. ABA vulnerability was compared within and across two groups of animals: those injected intraperitoneally with 30 mg/kg ketamine for three consecutive days (30mgKetx3) during the second ABA in late adolescence (ABA2) or with vehicle only (Vx3).

**Results:** Vx3 females and males exhibited individual differences in wheel running and weight retention during first ABA in mid-adolescence (ABA1), ABA2, and third ABA in adulthood (ABA3). Their wheel running correlated with anxiety-like behavior. During ABA1 and ABA3, weight gain of Vx3 females (but not males) after food consumption correlated negatively with food-anticipatory activity (FAA) preceding the feeding hours, indicating that females with higher levels of running restrict feeding more and persistently. This paradoxical relationship confirms earlier findings of ABA females without ketamine treatment, capturing the maladaptive behaviors exhibited by individuals diagnosed with anorexia nervosa. By contrast, 30mgKetx3 had an effect on both sexes of reducing hyperactivity during the feeding hours acutely and reducing anxiety-like behavior's contribution to running. For females, only, 30mgKetx3 acutely improved the extent of compensatory food consumption relative to FAA and improved weight retention during ABA3, 12 days post ketamine in adulthood.

**Discussion:** Sub-anesthetic ketamine evokes behavior-specific ameliorative effects for adult mice re-experiencing ABA, supporting the notion that multiple doses of ketamine may be helpful in reducing relapse among adults with anorexia nervosa.

**Public Significance Statement:** This study examined whether ketamine reduces anorexia-like behaviors in adult mice. Three daily sub-anesthetic ketamine injections suppress wheel running during and leading up to the hours of food availability and enable animals to compensate better for weight loss associated with excessive exercise by eating more. These findings suggest that ketamine may help adult females

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diagnosed with anorexia nervosa but also point to sex- and age-related differences in the action of ketamine.

**KEYWORDS**
activity-based anorexia, anorexia nervosa, anxiety, elevated plus maze, exercise, exploration, food-anticipatory activity, ketamine, mouse, wheel running

## 1 | INTRODUCTION

Anorexia nervosa is a mental illness comprised of the key symptoms of compulsive voluntary food restriction (FR), severe weight loss, and body dysmorphia (APA, 2013). In almost all cases, anorexia nervosa is also associated with excessive exercise that contributes to the severity of weight loss (Beadle et al., 2015; Beumont et al., 1994; Carrera et al., 2012; Davis et al., 1997; Davis et al., 1999; Hebebrand et al., 2003; Kron et al., 1978). Anorexia nervosa has a high rate of mortality (Arcelus et al., 2011; Birmingham et al., 2005; Nielsen et al., 1998; Sullivan, 1995), even surpassing that of major depressive disorders (Arcelus et al., 2011). Individual, family-based and cognitive behavioral therapies (Dalle Grave et al., 2016; Galsworthy-Francis & Allan, 2014) have been the most efficacious in re-directing and suppressing patients' maladaptive thinking patterns that perpetuate disordered eating. Anorexia nervosa is often associated with anxiety that predates the diagnosis (Dellava et al., 2010; Kaye et al., 2009), but anxiolytics have been disappointingly ineffective (Steinglass et al., 2014). Although the atypical antipsychotic, olanzapine, can reduce hyperactivity (Hillebrand et al., 2005) and appears associated with modest weight gain (Han et al., 2022), there is still no definitively effective pharmacotherapy (Crow, 2019). Recent developments suggest that sub-anesthetic doses of ketamine, an FDA-approved antidepressant, can ameliorate severity of the progression and relapse of anorexia nervosa for some, although not all, by reducing the maladaptive, compulsive thinking patterns (Calabrese et al., 2022; Martinotti et al., 2021; Mills et al., 1998; Schwartz et al., 2021; Scolnick et al., 2020). However, these ketamine data are in pilot phases, limited to open series administered to outpatients that are nearly weight-restored, with heterogeneity in co-morbidities and outcome. Schedules for ketamine administration also varied widely, in terms of the route, doses and number of administrations, with some that were combined with ketogenic diet. There is also paucity of knowledge regarding the mechanism of action of ketamine in treating anorexia nervosa and other mental illnesses, as well as the origin of individual differences in responsivity to ketamine (Gerhard et al., 2020; Li et al., 2010; Luscher et al., 2020).

Activity-based anorexia (ABA) is an animal model that captures all of the key symptoms of anorexia nervosa except for body dysmorphia. Hyperactivity, voluntary FR, elevated anxiety and severe weight loss can be evoked in the majority (~80%) of wildtype adolescent female mice by first acclimating them to a wheel in the cage, then restricting food access to 2 h/day (Aoki, 2020; Aoki et al., 2017; Aoki & Santiago, 2022; Beeler & Burghardt, 2021; Foldi et al., 2017; Wable, Chen, et al., 2015). These features make the mouse model suitable for investigating pharmacotherapy. A single sub-anesthetic dose of intraperitoneal (IP) ketamine was shown to ameliorate severity of ABA of adolescent female mice acutely and >22 days after the injection (Chen, Sherpa, & Aoki, 2018). However, the same dose administered during the second exposure to ABA in late adolescence (ABA2) exacerbated FR-evoked wheel running (food anticipatory activity, FAA) and weight loss, with no detectable protection from a third experience of ABA in adulthood (ABA3) (Aoki, 2020). This is disappointing from a translational point of view, since relapse is especially high among adults, compared to adolescents (Berends et al., 2018; Walsh, 2013). Thus, we asked how ketamine treatment could be tweaked to reduce ABA vulnerability of adult mice experiencing multiple cycles of ABA. In this study, first ABA in mid-adolescence (ABA1) was imposed during mid-adolescence to measure individual differences in ABA susceptibility at an age corresponding to most common first occurrence of anorexia nervosa. Then, ABA2 in the transition phase from late-adolescence to adulthood was imposed to test individual differences in responsiveness to ketamine in a relapse-like condition. Finally, ABA3 was imposed in adulthood to look at longer-term protective effects of ketamine to a relapse-like condition in adulthood.

In many studies exploring the action of ketamine as an antidepressant (Calabrese, 2019) and for treating anorexia nervosa in adulthood, ketamine has been administered as multiple doses (Calabrese et al., 2022; Mills et al., 1998; Ragnhildstveit et al., 2022; Scolnick et al., 2020). We reasoned that ABA mice approaching adulthood may also require multiple injections of ketamine, instead of a single injection (Aoki, 2020). Three parameters of ABA vulnerability were measured: weight loss, restricted food consumption and hunger-evoked alterations in wheel running. Results indicate that three doses of 30 mg/kg is effective for reducing all of these measures plus anxiety-like behavior in female mice and to lesser extent, also in males. Additionally, ketamine may have altered males' motivation for wheel running from anxiety to exploration.

## 2 | METHOD

*Animals*: All animals were bred at the animal facility of New York University, were of the C57BL6 strain, derived from 14 litters and

distributed across 7 cohorts. Please see Supplemental Materials for further details about the animals.

## 2.1 | The activity-based anorexia procedure and ketamine injections

Figure 1 shows the timeline of three cycles of ABA, applied to all males and females. A previous publication (Aoki, 2020) and Supplemental Materials and Methods provide details not included here. In brief, each cycle of ABA contained three phases: (1) acclimation to the wheel and wet food, during which time baseline wheel running, food consumption and body weights (referred to as weights from hereon) were assessed for each animal; (2) FR that began at 1 p.m. of FR1, in the presence of the wheel; (3) recovery, with ad libitum food access and no wheel. Low-profile wireless wheels (Med Associates, ENV-044) were fitted within each animal's cage. Ketamine was injected at 6 p.m. of ABA2's 2nd, 3rd, and 4th days of FR (i.e., FR2, FR3, FR4), 1 h before the feeding hours.

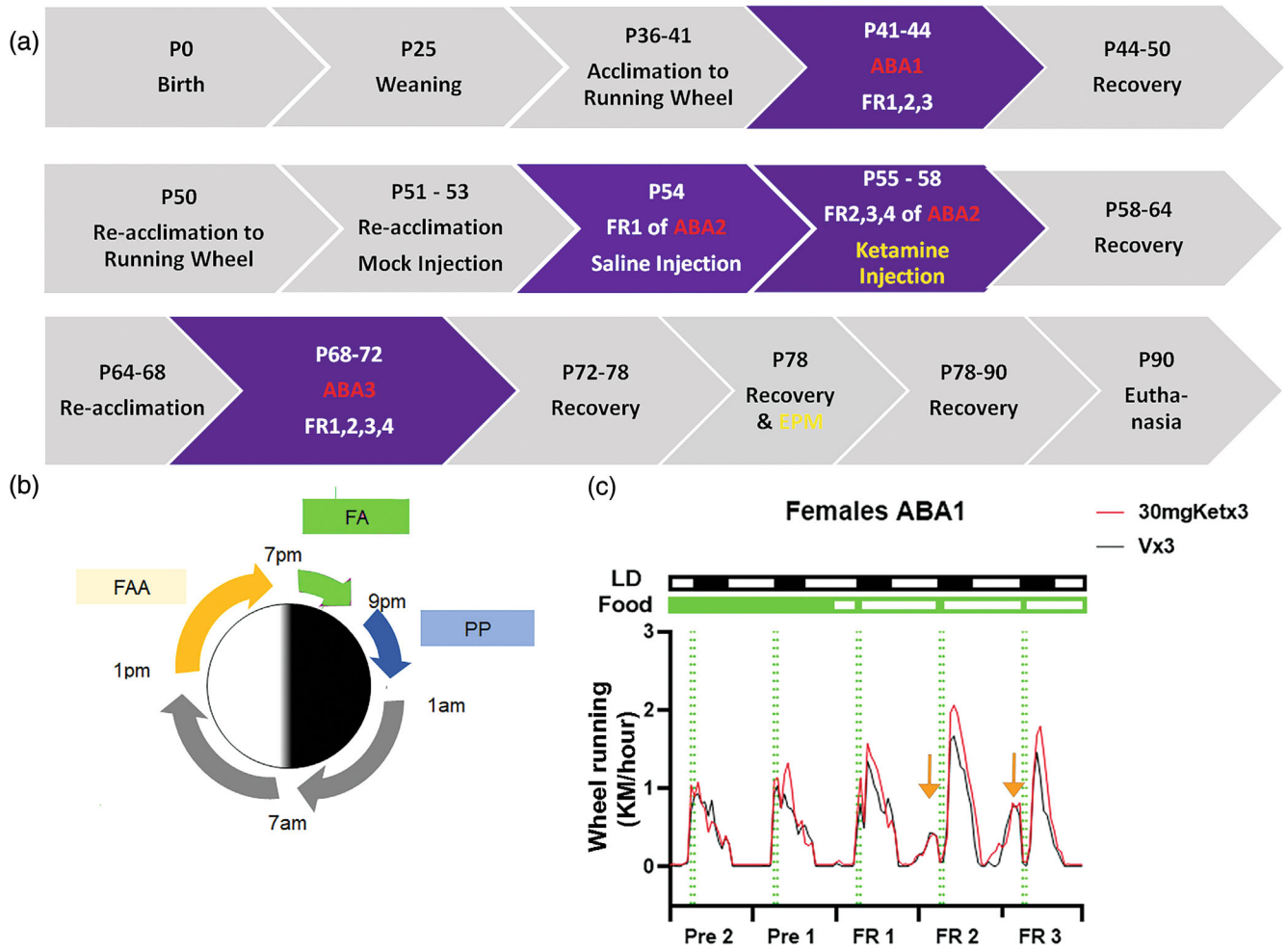


**FIGURE 1** Experimental schedule. Panel a: Timeline comprised of ABA1, ABA2, and ABA3, the ketamine injections and elevated plus maze test (EPM) for anxiety and exploration. The actual postnatal days varied by up to ±3 days except for euthanasia, which varied by up to ±9 days. FR1, 2, 3, and 4 refer to the 1st, 2nd, 3rd, and 4th day of food restriction (FR) which were strictly 3 days for ABA1 and strictly 4 days for ABA2 and ABA3. Drug injection time was strictly 6 p.m. The number of days for acclimation on the wheel and recovery from ABA varied by ±2 days. P = postnatal day. Adulthood is reached at around P60. P41-44 spans mid-adolescence and P55-58 spans late adolescence. Panel b: Circadian schedule during ABA. FAA = hours of food anticipatory activity; FA = 2 h of food availability; PP = post-prandial hours. Lights went out at 7 p.m. and on at 7 a.m. Panel c: Hourly wheel running in kilometers (KM) of the two female groups during ABA1, assigned to be Vx3 and 30mgKetx3 but without any difference in treatment yet, since ketamine versus vehicle treatments are administered during ABA2. The vertical green dotted lines depict the hours of 7 p.m. and 9 p.m., which aligned with the beginning and the end of feeding hours during the food restricted days FR1, FR2, and FR3. Hours of food availability for FR and non-FR days are indicated by the green bar ("Food"). On the days preceding FR (Pre 2 and Pre 1), food was available 24 h/day. LD indicates the timing of the light: dark cycle of the room. Note the progressive increase of FAA from FR2 to FR3 (orange arrows) and suppressed running during FA. The Supplemental Material section contains additional details and hourly wheel activities during ABA1, ABA2, and ABA3 of females and males as well as their body weights.

### 2.2 | Elevated plus maze

Elevated plus maze (EPM) was conducted to quantify anxiety- and exploration-like behaviors after recovery from ABA3 (detailed in Supplemental Material and Methods).

### 2.3 | Statistical analyses

Statistical analyses are detailed in Supplemental Materials and within the Results section.

## 3 | RESULTS

Previous results showed that both males and females exhibit ABA vulnerability but that the underlying mechanisms for gaining resilience differ by sex (Chen & Aoki, 2015; Chen, Actor-Engel, & Aoki, 2018). Moreover, responsiveness to ketamine has been shown to differ across the sexes (Ponton et al., 2022). Thus, data analysis was performed for males and females separately. Male data pertaining to weight, food consumption and running appear under Supplemental Materials. This study focused on treating animals with 30 mg/kg IP, the dose that ameliorated ABA when injected in mid-adolescence (Chen, Sherpa, & Aoki, 2018). Results from the dose of 10 mg/kg are preliminary and can be found under the Supplemental Material's Results section.

### 3.1 | 30mgKetx3 during late adolescence enhanced weight retention of female mice during ABA3 in adulthood

Body weights (weights) measured at 7 p.m., just prior to feeding, represent the lowest point for a given mouse on each experimental day. Weights measured at 9 p.m. reflect weight gain through feeding minus weight loss due to wheel running during the 2 h of food availability (FA) (Aoki, 2020; Du et al., 2022). Each animal's weight was normalized to its baseline, calculated as the average of the measurements just prior to the removal of food at 1 p.m. on FR1 and 1 day prior (Supplementary Figure 1). FR3 was analyzed to assess cumulative weight change of each ABA, while still allowing comparison with ABA1, which did not have FR4, because most animals undergoing ABA1 would be at a weight below the cutoff of 25%-below-baseline, if ABA continued beyond FR3 (Chowdhury et al., 2013).

Weights at 7 p.m. on FR3 of ABA1 and ABA2, relative to baseline were not significantly different between Vx3 and 30mgKetx3 ($p = .5048$, Figure 2a and $p = .6469$, Figure 2b, Table 1), but by ABA3, 13 days after the ketamine treatment, 30mgKetx3's weights were greater than Vx3's ($p = .0465$, Figure 2c and Table 1).

For the Vx3 group, within-group analysis indicated that weights at 7 p.m. on FR3 of ABA2 were significantly greater than at 7 p.m. on FR3 of ABA1, reflecting gain of resilience (Figure 2d; $p = .0038$, 0.035 ± 0.011 gain, Table 3a), as shown previously (Chowdhury et al., 2013). The gain of resilience was more consistent for the 30mgKetx3 than for the Vx3 group (Figure 2e; $p < .0001$, 0.031 ± 0.008 gain, Table 3b). This ketamine-mediated improvement was even more evident when comparing weights at 7 p.m. on FR3 of ABA3 versus ABA1 (0.033 ± 0.011 gain for Vx3, Figure 2d, $p = .0001$, Table 3a; 0.042 ± 0.008 gain for 30mgKetx3, Figure 2e, $p < .0001$, Table 3b).

### 3.2 | Weight loss trait was stable within individuals across the three ABA cycles of Vx3 females but altered by 30mgKetx3

Weights of Vx3 animals varied widely during ABA1, 2 and 3 (Figures 2a–c) but within-group paired t-tests indicated that almost all also exhibited slight improvements in weight retention when exposed to repeated ABA. Was an individual's weight retention consistent from ABA1 to ABA3, reflecting individual traits in responsiveness to FR (i.e., vulnerability vs. gain of resilience to the ABA-inducing environment)? If so, this stable trait might have been altered by ketamine delivered during ABA2 to yield better weight retention. Spearman correlation of weights at 7 p.m. on FR3 of ABA1 versus ABA3 was highly significant for the Vx3 group ($p = .0009$, Figure 2f). In sharp contrast to the Vx3 group, the 30mgKetx3 group's correlation value comparing ABA1 to ABA3 was low and not significant ($p = .2399$, Figure 2f), reflecting a trend towards a significant difference in correlation (two-tailed $p = .077$, Fisher z-score 1.77). This loss of correlation was due to some being heavier than predicted, based on their responsiveness to FR during ABA1. This indicates that most mice responded to the 30mgKetx3 treatment by improving their weights during the subsequent ABA induction beyond what could be expected from their ABA experience 3 weeks earlier, during adolescence and in the absence of ketamine.

At 9 p.m. on the same day, after feeding, the weights still correlated between ABA1 and ABA3 for the Vx3 group ($p = .0181$) (Figure 2g) but not for the 30mgKetx3 group ($p = .8201$), indicating a trend towards significant difference in the two correlations ($z = 1.6$; $p = 1095$). Note that the one individual with the lowest weight retention at 9 p.m. of ABA1 (79% of baseline) became the most resilient in ABA3 (97% of baseline) following 30mgKetx3 treatment.

### 3.3 | 30mgKetx3 reduced wheel running of females during the hours of food access acutely and during FAA with a delay

Food-anticipatory activity (FAA) increases as FR is repeated over multiple days, reflecting the cumulative weight loss that evokes progressive exacerbation of hunger-evoked hyperactivity (Aoki, 2020; Santiago et al., 2021) (Figure 1c). Conversely, wheel running during the hours of food availability (FA) reduces as FR is repeated, reflecting animals' ability to learn to eat instead of run during the limited hours of food availability (Aoki, 2020; Du et al., 2022; Santiago et al., 2021; Wu et al., 2014) (Figure 1c). Hunger-evoked wheel running was

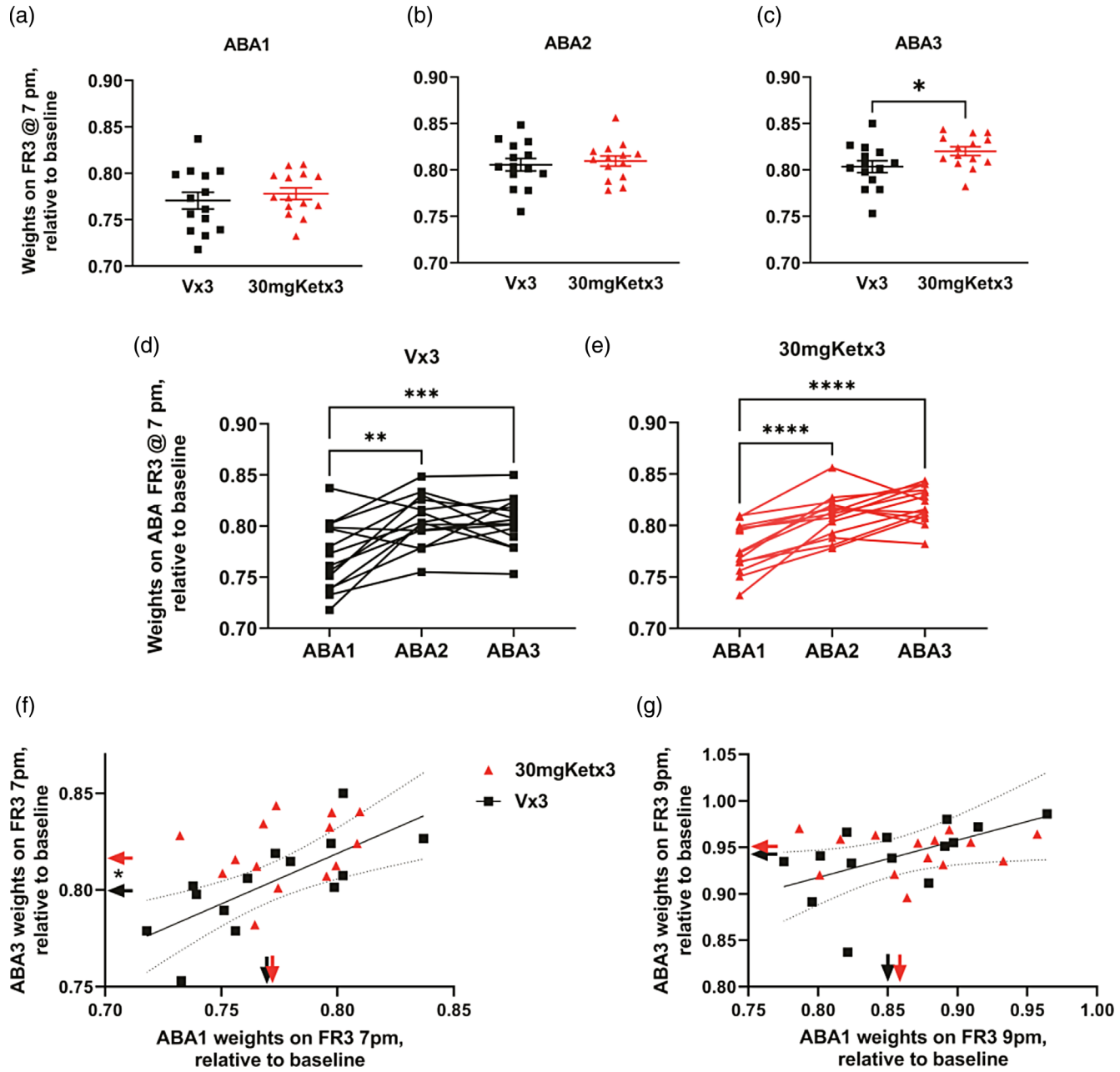


**FIGURE 2** Adult females' weight retention is improved significantly across 3 cycles of ABA and is improved further during ABA3 by 30mgKetx3. Panels a, b and c: For each animal, body weight (weight) at 7 p.m. on FR3 of ABA1 (panel a), ABA2 (panel b), and ABA3 (panel c) was normalized to baseline weights calculated as the average of weights at 1 p.m. on FR1 (just prior to food removal) and 1 day prior. Each data point represents the normalized value of one animal, either of the Vx3 (black square) or 30mgKetx3 group (red triangles). A statistically significant increase in the group mean weight emerged for the 30mgKetx3 group at ABA3 (panel c). ($N = 14$ for both groups; $p = .0465$). See Table 1 for mean ± SEM and 95% CIs. Panel d: Within-group comparisons of wheel running across 3 ABA cycles was conducted by Repeated measures one-way ANOVA, with Tukey's corrections for multiple comparisons. The Vx3 group showed significant improvements in weight retention at 7 p.m. of FR3 across the 3 cycles of ABA. Difference between means was 0.035 ± 0.011 gain comparing ABA2 versus ABA1 (** indicating $p = .0038$) and was 0.033 ± 0.011, comparing ABA3 versus ABA1 (*** indicating $p = .0001$). Panel e: Identical comparisons of 30mgKetx3 group showed greater improvements. Difference between means was 0.031 ± 0.008, comparing ABA2 versus ABA1 (**** indicating $p < .0001$) and was 0.042 ± 0.008, comparing ABA3 versus ABA1 ($p < .0001$). Panel f: The normalized weights obtained for each animal at 7 p.m. on FR3 of ABA1 correlated to the normalized weights at 7 p.m. of FR3 of ABA3 for the Vx3 group (Spearman $R = .8022$; $p = .0009$, $N = 14$) but not for the 30mgKetx3 group ($R = .3363$, $p = .2399$, $N = 14$). Five individuals of the 30mgKetx3 group exhibited weights during ABA3 that exceed the values predicted based on ABA1 weights. Panel g: The normalized weights obtained for each animal at 9 p.m. on FR3 of ABA1 correlated to the normalized weights at 9 p.m. of FR3 of ABA3 for the Vx3 group (Spearman $R = .6308$; $p = .0181$) but not for the 30mgKetx3 group ($R = .0681$; $p = .8201$). Three out of the 14 of the 30mgKetx3 exhibited weights during ABA3 that exceeded the values predicted based on ABA1 BW values. For both panels f and g: the solid lines indicate significant trends, while the dotted curves depict 95% confidence ranges. Arrows depict the group mean values (black for Vx3; red for 30mgKetx3).

**TABLE 1** Group comparisons of females' BW, activity during FR days and EPM behavior.

| | Female Vx3 ($N = 14$) | Female 30mgKetx3 ($N = 14$) | *p*-value | Mean difference | 95% CI |
|---|---|---|---|---|---|
| BW ABA1 FR3 @ 7 p.m., relative to baseline | 0.771 ± 0.009 | 0.778 ± 0.006 | .5048 | 0.007462 ± 0.01103 | −0.01522 to 0.03014 |
| BW ABA2 FR3 @ 7 p.m., relative to baseline | 0.806 ± 0.007 | 0.810 ± 0.006 | .6469 | 0.004002 ± 0.008634 | −0.01374 to 0.02175 |
| BW ABA3 FR3 @ 7 p.m., relative to baseline | 0.804 ± 0.006 | 0.820 ± 0.005 | .0465 | 0.01665 ± 0.007963 | 0.0002800 to 0.03302 |
| BW ABA3 FR4 @ 9 p.m., relative to baseline | 0.929 ± 0.015 | 0.936 ± 0.008 | .6945 | 0.006733 ± 0.01696 | −0.02812 to 0.04159 |
| Wheel activity 6 p.m.–7 p.m. (Avg of ABA2 FR234) | 0.546 ± 0.102 | 0.320 ± 0.066 | .0715 | 0.2258 ± 0.1200 | −0.02132 to 0.4729 |
| Wheel dwell time 6 p.m.–7 p.m. (Avg of ABA2 FR234) | 24.49 ± 2.520 | 22.31 ± 2.718 | .5638 | 2.178 ± 3.723 | −5.490 to 9.845 |
| FAA increase ABA1 (Avg of FR23)-(Avg of baseline) | 2.497 ± 0.570 | 2.364 ± 0.530 | .8659 | 0.1328 ± 0.7786 | −1.468 to 1.733 |
| FAA increase ABA2 (Avg of FR234)-(Avg of baseline) | 1.780 ± 0.402 | 1.642 ± 0.4499 | .8217 | 0.1382 ± 0.6069 | −1.112 to 1.388 |
| FAA increase ABA2 (Avg of FR34)-(Avg of baseline) | 2.312 ± 0.455 | 2.449 ± 0.671 | .8691 | −0.1370 ± 0.8228 | −1.831 to 1.558 |
| FAA increase ABA3 (Avg of FR234)-(Avg of baseline) | 2.293 ± 0.5358 | 0.8974 ± 0.3217 | .0343 | 1.396 ± 0.6249 | 0.1113 to 2.680 |
| FA increase ABA1 (Avg of FR23)-(Avg of baseline) | −1.662 ± 0.199 | −1.928 ± 0.201 | .3556 | 0.2660 ± 0.2828 | −0.3154 to 0.8474 |
| FA increase ABA2 (Avg of FR234)-(Avg of baseline) | −1.501 ± 0.234 | −2.464 ± 0.268 | .0125 | 0.9632 ± 0.3578 | 0.2263 to 1.700 |
| FA increase ABA3 (Avg of FR234)-(Avg of baseline) | −1.768 ± 0.266 | −2.002 ± 0.319 | .5781 | 0.2341 ± 0.4157 | −0.6203 to 1.088 |
| PP increase ABA1 (Avg of FR23)-(Avg of baseline) | 1.507 ± 0.522 | 1.973 ± 0.396 | .4835 | −0.4655 ± 0.6548 | −1.812 to 0.8805 |
| PP increase ABA2 (Avg of FR234)-(Avg of baseline) | 2.110 ± 0.499 | 2.046 ± 0.447 | .9244 | 0.06400 ± 0.6681 | −1.312 to 1.440 |
| PP increase ABA3 (Avg of FR234)-(Avg of baseline) | 1.929 ± 0.323 | 2.043 ± 0.625 | .8727 | −0.1139 ± 0.7038 | −1.561 to 1.333 |
| Total increase ABA1 (Avg of FR23)-(Avg of baseline) | 3.511 ± 1.326 | 5.412 ± 1.063 | .2735 | −1.902 ± 1.700 | −5.395 to 1.592 |
| Total increase ABA2 (Avg of FR234)-(Avg of baseline) | 3.438 ± 1.258 | 3.638 ± 0.998 | .9230 | −0.1556 ± 1.594 | −3.438 to 3.127 |
| Total increase ABA3 (Avg of FR234)-(Avg of baseline) | 2.092 ± 0.8407 | 1.601 ± 1.295 | .7530 | 0.4909 ± 1.544 | −2.682 to 3.664 |
| EPM open arm frequency, % of total entries | 24.20 ± 3.762 | 26.58 ± 3.104 | .6335 | 2.375 ± 4.920 | −7.757 to 12.51 |

*Note*: Two-tailed unpaired *t*-tests comparing the 30mgKetx3 versus Vx3 groups of females were performed. All values are shown as mean ± SEM. Data from FR1 were excluded from averaging since animals are not yet experiencing FR-evoked hunger on FR1. ABA1 had 3 days of FR (FR1, FR2, FR3), from which body weight (BW) and running data during FR2 and FR3 were averaged. ABA2 and ABA3 had 4 days of FR (FR1, FR2, FR3, FR4), from which BW and running data during the latter 3 days were averaged. Baseline BW and running were assessed during the 2 days preceding FR, then averaged and subtracted from the averaged FR values. Running activity was binned as follows: FAA = food anticipatory activity, from 1 p.m. to 7 p.m.; FA = during the 2 h of food availability, from 7 p.m. to 9 p.m.; PP = post prandial, from 9 p.m. to 1 a.m. of the next day. Total = sum of running over 24 h, starting from 1 p.m. $N = 13$ for analyses pertaining to Vx3 group's ABA2, due to loss of data associated with wheel count data acquisition. Wheel activity immediately after the administration of 30 mg of ketamine or vehicle, from 6 p.m. to 7 p.m. of ABA2 FR234 was also assessed. Wheel activity is expressed as KM, wheel dwell time is expressed as minutes. *P*-values highlighted in red are significant (<.05).

assessed during the FR-days, then averaged across FR2 to 4, excluding FR1 data when animals were not yet underweight (Supplementary Figure 1). Wheel activity was individualized by calculating the change evoked by hunger, in comparison to baseline during the 2 days preceding FR (FAA-minus-baseline and FA-minus-baseline, abbreviated as "FAA-baseline" and "FA-baseline").

During the food access periods of ABA2 (FA, 7 p.m.–9 p.m.), that is, 1–3 h after the ketamine injection at 6 p.m., the 30mgKetx3 group reduced wheel running significantly, compared to the Vx3 group ($p = .0125$) (Figure 3b and Table 1). This group difference was not observed during ABA1, before the ketamine treatment ($p = .3556$, Figure 3a and Table 1). Within-group comparison of ABA2 versus

**TABLE 2** Group comparisons of males' BW, activity during FR days and EPM behavior.

| | Male Vx3 ($N = 9$) | Male 30mgKetx3 ($N = 8$) | *p*-value | Mean difference | 95% CI |
|---|---|---|---|---|---|
| BW ABA1 FR3 @ 7 p.m., relative to baseline | 0.789 ± 0.009 | 0.763 ± 0.015 | .1612 | −0.02627 ± 0.01783 | −0.06427 to 0.01172 |
| BW ABA2 FR3 @ 7 p.m., relative to baseline | 0.834 ± 0.006 | 0.820 ± 0.009 | .1782 | −0.01470 ± 0.01041 | −0.03688 to 0.007482 |
| BW ABA3 FR3 @ 7 p.m., relative to baseline | 0.847 ± 0.007 | 0.838 ± 0.006 | .3319 | −0.008847 ± 0.008824 | −0.02765 to 0.009960 |
| BW ABA3 FR4 @ 9 p.m., relative to baseline | 0.951 ± 0.006 | 0.942 ± 0.005 | .3363 | −0.008759 ± 0.008817 | −0.02755 to 0.01003 |
| Wheel activity 6 p.m.–7 p.m. (Avg of ABA2 FR234) | 0.2955 ± 0.104 | 0.293 ± 0.057 | .9869 | 0.002052 ± 0.1227 | −0.2595 to 0.2636 |
| Wheel dwell time 6 p.m.–7 p.m. (Avg of ABA2 FR234) | 15.37 ± 4.171 | 22.08 ± 3.338 | .2358 | −6.713 ± 5.435 | −18.30 to 4.871 |
| FAA increase ABA1 (Avg of FR23)-(Avg of Baseline) | 1.799 ± 0.403 | 3.094 ± 0.5239 | .0658 | −1.295 ± 0.6491 | −2.688 to 0.09691 |
| FAA increase ABA2 (Avg of FR234)-(Avg of Baseline) | 0.961 ± 0.444 | 2.112 ± 0.632 | .150 | −1.151 ± 0.7591 | −2.769 to 0.4667 |
| FAA increase ABA2 (Avg of FR34)-(Avg of Baseline) | 1.454 ± 0.664 | 3.131 ± 0.938 | .1583 | −1.677 ± 1.129 | −4.083 to 0.7301 |
| FAA increase ABA3 (Avg of FR234)-(Avg of Baseline) | 0.994 ± 0.442 | 1.249 ± 0.449 | .6913 | −0.2558 ± 0.6317 | −1.602 to 1.091 |
| FA increase ABA1 (Avg of FR23)-(Avg of Baseline) | −1.440 ± 0.280 | −1.581 ± 0.220 | .6944 | −0.1410 ± 0.3519 | −0.8910 to 0.6090 |
| FA increase ABA2 (Avg of FR234)-(Avg of Baseline) | −1.825 ± 0.300 | −2.313 ± 0.299 | .2692 | 0.4877 ± 0.4251 | −0.4183 to 1.394 |
| FA increase ABA3 (Avg of FR234)-(Avg of Baseline) | −1.505 ± 0.292 | −1.512 ± 0.333 | .9874 | 0.007076 ± 0.4403 | −0.9314 to 0.9455 |
| PP increase ABA1 (Avg of FR23)-(Avg of Baseline) | 2.269 ± 0.456 | 2.954 ± 0.659 | .3976 | −0.6847 ± 0.7863 | −2.361 to 0.9914 |
| PP increase ABA2 (Avg of FR234)-(Avg of Baseline) | 2.814 ± 0.430 | 2.611 ± 0.493 | .7599 | 0.2027 ± 0.6511 | −1.185 to 1.591 |
| PP increase ABA3 (Avg of FR234)-(Avg of Baseline) | 2.519 ± 0.361 | 1.638 ± 0.289 | .0807 | 0.8810 ± 0.4704 | −0.1216 to 1.884 |
| Total increase ABA1 (Avg of FR23)-(Avg of Baseline) | 6.558 ± 1.270 | 8.502 ± 1.601 | .3512 | −1.946 ± 2.022 | −6.255 to 2.364 |
| Total increase ABA2 (Avg of FR234)-(Avg of Baseline) | 3.542 ± 0.798 | 4.445 ± 0.769 | .4305 | −0.9033 ± 1.115 | −3.280 to 1.473 |
| Total increase ABA3 (Avg of FR234)-(Avg of Baseline) | 3.941 ± 1.230 | 2.124 ± 0.614 | .2233 | 1.817 ± 1.430 | −1.232 to 4.865 |
| EPM open arm frequency, % of total entries | 25.63 ± 3.069 | 20.54 ± 1.926 | .2114 | −5.093 ± 3.889 | −13.43 to 3.249 |

*Note*: Two-tailed unpaired *t*-tests comparing the 30mgKetx3 versus Vx3 groups of males were performed. All values are shown as mean ± SEM. Data from FR1 were excluded from averaging since animals are not yet experiencing FR-evoked hunger on FR1. ABA1 had 3 days of FR (FR1, FR2, FR3), from which body weight (BW) and running data during FR2 and FR3 were averaged. ABA2 and ABA3 had 4 days of FR (FR1, FR2, FR3, FR4), from which BW and running data during the latter 3 days were averaged. Baseline BW and running were assessed during the 2 days preceding FR, then averaged and subtracted from the averaged FR values. Running activity was binned as follows: FAA = food anticipatory activity, from 1 p.m. to 7 p.m.; FA = during the 2 h of food availability, from 7 p.m. to 9 p.m.; PP = post prandial, from 9 p.m. to 1 a.m. of the next day. Total = sum of running over 24 h, starting from 1 p.m. Wheel activity immediately after the administration of 30 mg of ketamine or vehicle, from 6 p.m. to 7 p.m. of ABA2 FR234 was also assessed. Wheel activity is expressed as KM, wheel dwell time is expressed as minutes.

ABA1 also showed significant reduction of each animal's running during FA for the 30mgKetx3 group (−0.79 ± 0.22 km, $p = .0104$, Figure 3e, Table 3b) but not for the Vx3 group (0.07 ± 0.23 km, $p = .952$ Figure 3d, Table 3a), indicating an acute ketamine effect. There was no group difference during ABA3 ($p = .5781$, Figure 3c and Table 1), and no significant within-group change from ABA2 to ABA3 for the 30mgKetx3 group (0.55 ± 0.32 km, $p = .234$, Table 3b) or the Vx3 group (0.02 ± 0.36 km, $p = .99$, Table 3a).

**TABLE 3** Comparisons of body weight (BW) and wheel activity across the three ABA periods of the 30mgKetx3 female group (Table 3a) and Vx3 female group (Table 3b).

a. ANOVA comparisons of female Vx3's BW and wheel activity across ABA periods ($N = 14$)

| | Mean 1 | Mean 2 | *p*-value | Mean difference | 95% CI |
|---|---|---|---|---|---|
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA2 | 0.7705 ± 0.009060 | 0.8056 ± 0.006655 | .0038 | −0.03510 ± 0.008711 | −0.05810 to −0.01210 |
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA3 | 0.7705 ± 0.009060 | 0.8035 ± 0.006418 | .0003 | −0.03300 ± 0.006128 | −0.04918 to −0.01682 |
| BW FR3 @ 7 p.m., relative to baseline: ABA2 vs. ABA3 | 0.8056 ± 0.006655 | 0.8035 ± 0.006418 | .9404 | 0.002101 ± 0.006278 | −0.01447 to 0.01868 |
| FA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA2 | −1.585 ± 0.1840 | −1.654 ± 0.1914 | .9519 | 0.06950 ± 0.2318 | −0.5566 to 0.6956 |
| FA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA3 | −1.585 ± 0.1840 | −1.631 ± 0.2926 | .9613 | 0.04588 ± 0.1712 | −0.4165 to 0.5082 |
| FA increase (Avg of FR)-(Avg of baseline): ABA2 vs. ABA3 | −1.654 ± 0.1914 | −1.631 ± 0.2926 | .9976 | −0.02361 ± 0.3605 | −0.9972 to 0.9500 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA2 | 2.632 ± 0.5983 | 1.780 ± 0.4021 | .5317 | 0.8520 ± 0.7741 | −1.213 to 2.917 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA3 | 2.632 ± 0.5983 | 2.113 ± 0.5450 | .7690 | 0.5187 ± 0.7430 | −1.463 to 2.501 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA2 vs. ABA3 | 1.780 ± 0.4021 | 2.113 ± 0.5450 | .7896 | −0.3333 ± 0.5039 | −1.678 to 1.011 |

b. ANOVA comparisons of female 30mgKetx3's BW and wheel activity across ABA periods ($N = 14$)

| | Mean 1 | Mean 2 | *p*-value | Mean difference | 95% CI |
|---|---|---|---|---|---|
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA2 | 0.7780 ± 0.006298 | 0.8096 ± 0.005500 | <.0001 | −0.03164 ± 0.005059 | −0.04500 to −0.01828 |
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA3 | 0.7780 ± 0.006298 | 0.8202 ± 0.004713 | <.0001 | −0.04218 ± 0.006752 | −0.06001 to −0.02436 |
| BW FR3 @ 7 p.m., relative to baseline: ABA2 vs. ABA3 | 0.8096 ± 0.005500 | 0.8202 ± 0.004713 | .1894 | −0.01054 ± 0.005668 | −0.02551 to 0.004421 |
| FA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA2 | −1.854 ± 0.2014 | −2.645 ± 0.2130 | .0104 | 0.7918 ± 0.2233 | 0.1960 to 1.388 |
| FA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA3 | −1.854 ± 0.2014 | −2.095 ± 0.3299 | .8327 | 0.2411 ± 0.4151 | −0.8663 to 1.349 |
| FA increase (Avg of FR)-(Avg of baseline): ABA2 vs. ABA3 | −2.645 ± 0.2130 | −2.095 ± 0.3299 | .2338 | −0.5507 ± 0.3180 | −1.399 to 0.2977 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA2 | 2.364 ± 0.5303 | 1.642 ± 0.4499 | .5250 | 0.7221 ± 0.6506 | −0.9958 to 2.440 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA3 | 2.364 ± 0.5303 | 0.8974 ± 0.3217 | .0543 | 1.466 ± 0.5650 | −0.02558 to 2.958 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA2 vs. ABA3 | 1.642 ± 0.4499 | 0.8974 ± 0.3217 | .1154 | 0.7443 ± 0.3440 | −0.1640 to 1.653 |

*Note*: One-way repeated measures ANOVA were performed. All values are shown as mean ± SEM. Data from FR1 was excluded from averaging since animals are not yet experiencing FR-evoked hunger on FR1. ABA1 had 3 days of FR (FR1, FR2, FR3), from which body weight (BW) and running data during FR2 and FR3 were averaged. ABA2 and ABA3 had 4 days of FR (FR1, FR2, FR3, FR4), from which BW and running data during the latter 3 days were averaged. Baseline BW and running were assessed during the 2 days preceding FR, then averaged and subtracted from the FR averaged values. Running activity was binned as follows: FAA = food anticipatory activity, from 1 p.m. to 7 p.m.; FA = during the 2 h of food availability, from 7 p.m. to 9 p.m. One of 14 females in the 30mgKetx3 group and one of 14 females in the Vx3 group did not decrease their running during FA of ABA2, justifying their exclusion from the FA analysis as described in the Results section. There was also a loss of data during Vx3 group's ABA2 associated with wheel count acquisition, which impacted one animal. Thus, $N = 13$ for 30mgKetx3 in FA tests, $N = 12$ for Vx3 in FA tests, and $N = 13$ for Vx3 in FAA tests. Wheel activity is expressed as KM. *P*-values highlighted in red are significant (<.05).

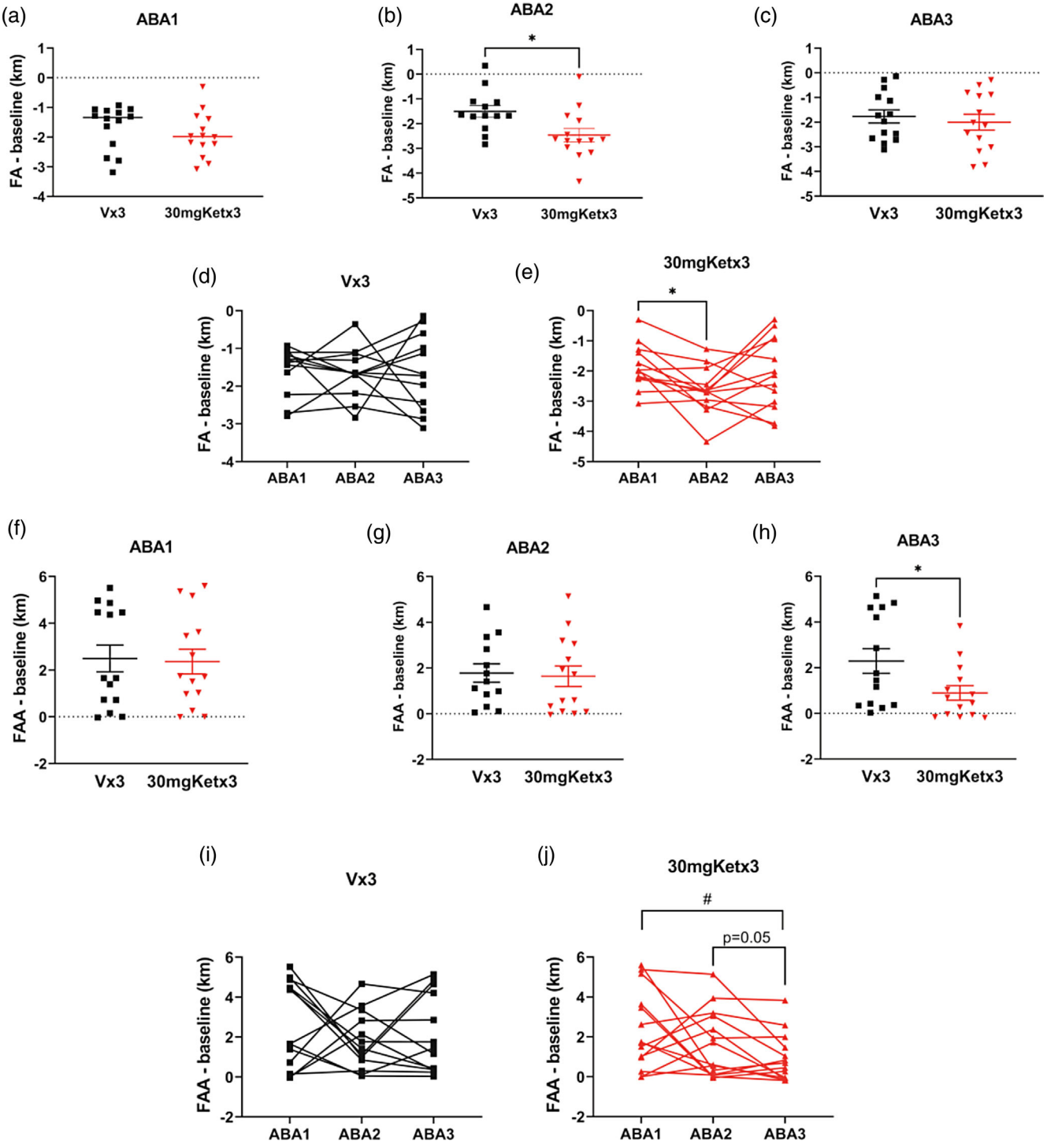


**FIGURE 3** 30mgKetx3 has an acute effect upon females of reducing wheel running during the subsequent 2 h of food availability (FA) of ABA2 and a delayed effect of reducing FAA during ABA3. Each data point represents the averaged value of one animal. Black squares represent data of the Vx3 group. Red triangles represent data of the 30mgKetx3 group. "FA-baseline" in panels a–e represent average wheel running during the 2 h of food availability on FR days excluding FR1, minus baseline running on days prior to FR. "FAA-baseline" in panels f–j represent averaged increase of food-anticipatory activity (FAA) during the 6 h leading up to food availability on FR days excluding FR1, minus baseline wheel running during the 2 days preceding FR. Panels a, b, c, f, g, and h show results of unpaired t-tests of comparisons across groups. Panels d, e, i, and j show results from within-group repeated measures one-way ANOVA, comparing group mean wheel running across three ABA cycles, with Tukey's corrections for multiple comparisons. FA of the 30mgKetx3 reduced by 0.79 ± 0.22 km from ABA2 to ABA1 ($p = .0104$) but was unchanged for the Vx3 group (0.07 ± 0.23 km). FAA of the 30mgKetx3 was reduced by 1.5 ± 0.565 km ($p = .054$), comparing ABA3 versus ABA1 and 0.75 ± 0.34 km ($p = .11$), comparing ABA3 versus ABA2. The difference of the means of FAA of Vx3, comparing ABA3 versus ABA1 (0.51 ± 0.74 km, $p = .77$) or comparing ABA3 versus ABA2 (0.33 ± 0.50 km, $p = .79$) were not significant. Significant group differences are indicated by "*". # indicates a trend. One outlier from the 30mgKetx3 and one outlier from the Vx3 were removed for the repeated measures one-way ANOVA comparison of FA.

As for FAA, 30mgKetx3 yielded no detectable acute effect during the last hour of FAA on ABA2 (6 p.m.–7 p.m.), immediately following the ketamine injection ($p = .0715$, comparing KM run of Vx3 to 30mgKetx3, averaged across FR2 to FR4; $p = .5638$, t = .5849, comparing their dwell time on the wheel, Table 1) or during the entire hours of FAA (1 p.m.–7 p.m.) of ABA2, averaged across FR2-4 ($p = .8217$, Figure 3g, Table 1) or the last 2 days (ABA2's FR3 and FR4, averaged, $p = .8691$, Table 1). This absence of locomotor effect during the first hour after injection (6 p.m.–7 p.m.) is as expected, since 30 mg/kg of ketamine is subanesthetic, compared to the anesthetic dose of 100 mg/kg for rodents.

Remarkably, 30mgKetx3 generated significant reduction of FAA during ABA3, 12 days later, relative to the Vx3 group ($p = .0343$, averaged across FR2-4, Figure 3f, Table 1). Within-group comparisons (Table 3a, b) confirmed this pattern of a more consistent, greater magnitude reduction of FAA for the 30mgKetx3 group, when compared between ABA1 versus ABA3 (Figure 3i for Vx3, $p = .769$; Figure 3j for 30mgKetx3, −1.5 ± 0.56 km, $p = .05$) or ABA2 versus ABA3 (Figure 3i for Vx3, $p = .790$; Figure 3j for 30mgKetx3, −0.74 ± 0.34 km, $p = .11$).

Some other research groups calculate FAA as a shorter period preceding feeding (e.g., 2.5 or 3 h (Assali et al., 2021; Wu et al., 2014). Our conclusion regarding the reduction of FAA by 30mgKetx3 remains unchanged, when defining FAA as 3 h prior to feeding (data not shown).

Other measurements of wheel activity–during the post-prandial hours (PP, 9 p.m.–1 a.m.) and the sum of 24 h, averaged across the FR2-4 (total) revealed no group difference (Table 1).

### 3.4 | 30mgKetx3 influenced the link between running and feeding of females

As indicated in the two previous figures, both weights and FAA varied widely among the 28 females. Did animals compensate for the cumulative weight loss (Supplementary Figure 1) accompanying the

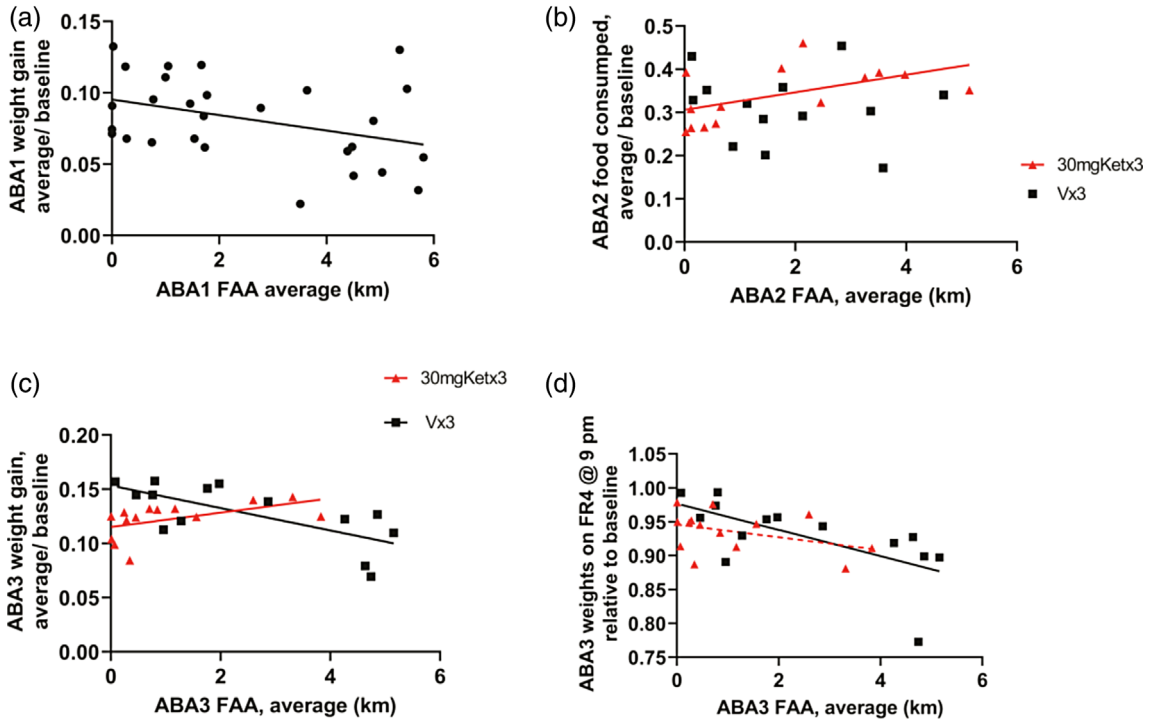


**FIGURE 4** Females' food-anticipatory activity (FAA) correlations to weight and food consumption are altered acutely and long-term by 30mgKetx3. Panel a: During ABA1, FAA, averaged across FR2 and FR3, correlates significantly and negatively ($R = -.3796$; $p = .0463$; $N = 28$) with averaged weight gain following the 2 h of food availability from 7 p.m. to 9 p.m., relative to baseline weights on the 2 days immediately preceding FR. The negative correlation indicates that those individuals exhibiting the most excessive hyperactivity are the same individuals that gained the least weight. Panel b: During ABA2, FAA of the 30mgKetx3 group, averaged across FR2, FR3, and FR4, correlates positively with kcal food consumed on those days, relative to baseline. Although far from equal in the amount of food consumed during the ad libitum days, the 30mgKetx3 group showed a significantly positive correlation between food consumed and extent of wheel running during FAA of the preceding 6 h ($p = .0436$, $R = .5455$). By contrast, the Vx3 group showed no correlation in feeding relative to wheel running ($p = .6448$, $R = -.1415$, $N = 13$). Panel c: During ABA3, FAA of Vx3 group correlated significantly and negatively with weight gained by the end of the feeding period, averaged across FR2, FR3, and FR4 (Spearman $R = -.6527$; $p = .0136$), indicating that the same animals that run excessively exhibited the least adaptive compensatory feeding after exercising. By contrast, FAA of the 30mgKetx3 group showed the conversion of the correlation from a negative value to a positive value ($R = .5692$; $p = .0366$; $z = 3.35$, $p = .0008$ indicating significant difference in correlation from the Vx3 group), indicating that they compensated for weight loss due to excessive running by eating more. Panel d: The two ABA vulnerability traits—low weight at 9 p.m. on FR4 (the last day of FR) of ABA3 and FAA—correlated significantly for the Vx3 group ($p = .0043$, $R = -.7275$), but this correlation was weakened for the 30mgKetx3 group ($p = .1119$; $R = -.4462$). Weight comparison: $p = .7$, 93% for Vx3 versus 97% for 30mgKetx3.

progressive FAA increase (Figure 1c and Supplementary Figure 1) by eating more? To answer this question, FAA was compared to weight gain from the feeding period.

Averaged FAA during ABA1 correlated significantly and negatively with weight gain during feeding ($p = .0463$, Figure 4a), suggesting that hyperactive mice were the same individuals that failed to compensate for weight loss through feeding or running less during the hours of food access (FA). This is the central behavioral paradox of ABA and anorexia nervosa and has led to the theory that hyperactivity and suppressed feeding both originate from heightened anxiety (Wable, Min, et al., 2015).

During ABA2's FR2-4, the Vx3 group showed no correlation between averaged FAA and food consumption, relative to baseline ($p = .645$, Figure 4b). By contrast, the 30mgKetx3 group showed a significantly positive correlation between FAA and food consumption ($p = .0436$, Figure 4b), with a trend towards significant difference in correlation ($z = 1.73$; $p = .08$) suggesting that ketamine improved animals' compensatory feeding, relative to hyperactivity during FAA.

During ABA3, the negative correlation between FAA and weight gain seen during ABA1 persisted for the Vx3 group ($p = .0136$, Figure 4c), indicating the persistence of anorexia-like maladaptive hyperactivity associated with poor weight gain during the feeding hours. By contrast, the 30mgKetx3 group showed a significant correlation in the opposite, positive direction ($p = .0366$, Figure 4c), with a statistically significant difference in correlation ($z = 3.35$, $p = .0008$). This indicates a carry-over ameliorative effect in ABA3 of ketamine administered during ABA2. Specifically, ketamine-treated animals compensated better for heightened energy expenditure associated with FAA hyperactivity by eating more and/or running less during the subsequent hours of food availability. The positive correlation also means that those animals that ran the most during FAA were the same individuals that ate the most during the feeding hours, reflecting a dissociation of two maladaptive behaviors—running and restrictive feeding. This dissociation among the 30mgKetx3 animals reflects departure from the maladaptive anorexic behavior.

By 9 p.m. of FR4 of ABA3, when the feeding hour ended for the last day of FR, the two groups showed equivalently good weight restoration, relative to baseline (93% for Vx3; 94% for 30mgKetx3; $p = .6945$, Table 1). Despite this, we observed a persistent negative correlation between FAA and weight gain among the Vx3 group ($p = .0043$, Figure 4d), indicating that the most hyperactive animals were the least able to return to healthy weights. By contrast, this correlation of the two maladaptive traits was weakened for the 30mgKetx3 group ($p = .1119$) (Figure 4d), without evoking significant change in correlation (z-score 1.04; $p = .2983$), suggesting that ketamine weakened the link between excessive running and cumulative weight loss 10–17 days later.

These changes in weight, FA and FAA induced by 30mgKetx3 were not observed at a lower dose of 10mgKetx3 (Supplementary Material).

### 3.5 | 30mgKetx3 acutely removed the contribution of anxiety to running among females

Food restriction-evoked increase in anxiety-like behavior of females, measured using EPM (elevated plus maze) has been shown to correlate with running (Wable, Min, et al., 2015). This anxiety-like behavior persists even after weight restoration (Chen et al., 2017) and continues to contribute towards increased wheel running during ABA2 (Aoki & Santiago, 2022). We built upon these previous findings while limiting EPM to one time point, to avoid animals' habituation to the open arms, which reduces test sensitivity (Schrader et al., 2018). That new time point was after recovery from ABA3, to investigate whether reduced anxiety contributes to reduced hyperactivity.

There was no group difference of EPM time in the open arm (not shown) or frequency of entries into the open arm (Frequency: $p = .6335$, Figure 5a, Table 1). However, the frequency to enter the open arm correlated significantly and negatively with total running specifically during ABA2 only for the Vx3 females ($p = .0052$, $N = 13$ for Vx3; $p = .4649$ for 30mgKetx3, Figure 5c; $z = 2.61$, $p = .0091$ indicating significant difference in correlation). This shows that Vx3 female mice that were most hyperactive during ABA2 (late adolescence) had the highest anxiety-like behavior in adulthood, and that ketamine acutely disrupted the maladaptive link from anxiety to running. For both treatment groups, the two maladaptive behaviors did not correlate during ABA1 (Figure 5b, $p = .8503$ for Vx3; $p = .5033$ for 30mgKetx3) or ABA3 (Figure 5d, $p = .5958$ for Vx3; $p = .8045$ for 30mgKetx3).

### 3.6 | 30mgKetx3 altered the dominant motivation for wheel running among males from anxiety to exploration during ABA3

Although 30mgKetx3 only subtly decreased maladaptive behaviors of weight loss (Supplementary Figure 2, Tables 2 and 4a,b) or hyperactivity (Supplementary Figure 3, Tables 2 and 4a,b), one remarkable effect of this treatment was an alteration in the link between behavior measured by EPM and wheel running. Like the females, the 30mgKetx3 and Vx3 groups were not significantly different in their frequency to enter the open arm ($p = .2114$, Figure 5e, Table 2). Unlike the females, correlations between anxiety-like behavior and wheel running were not evident among either group of males during ABA2 (Figure 5f, g) but emerged during ABA3. Among Vx3 males, the frequency to enter the open arm correlated strongly and negatively with the averaged total wheel running ($p = .0099$, Figure 5h). Strikingly unlike Vx3 males, 30mgKetx3 males exhibited a strong *positive* correlation ($p = .0047$, Figure 5h). Frequency to enter open arms of the EPM (i.e., 1 minus frequency to enter the closed arm) is considered to reflect exploratory behavior (Chowdhury et al., 2021; Pellow & File, 1986). Thus, the switch in the *R*-values from negative to positive suggests that wheel running of the Vx3 males was driven primarily by anxiety (staying in the closed arms) evoked by FR, as was reported for females (Wable, Min, et al., 2015) but was converted by 30mgKetx3 to become manifestation of hunger-evoked *exploratory* behavior (entering the open arms more).

## 4 | DISCUSSION

Anorexia nervosa is a mental illness with high rates of mortality and relapse, especially in adulthood (Arcelus et al., 2011), yet is without clear consensus for pharmacotherapy (Crow, 2019). This study

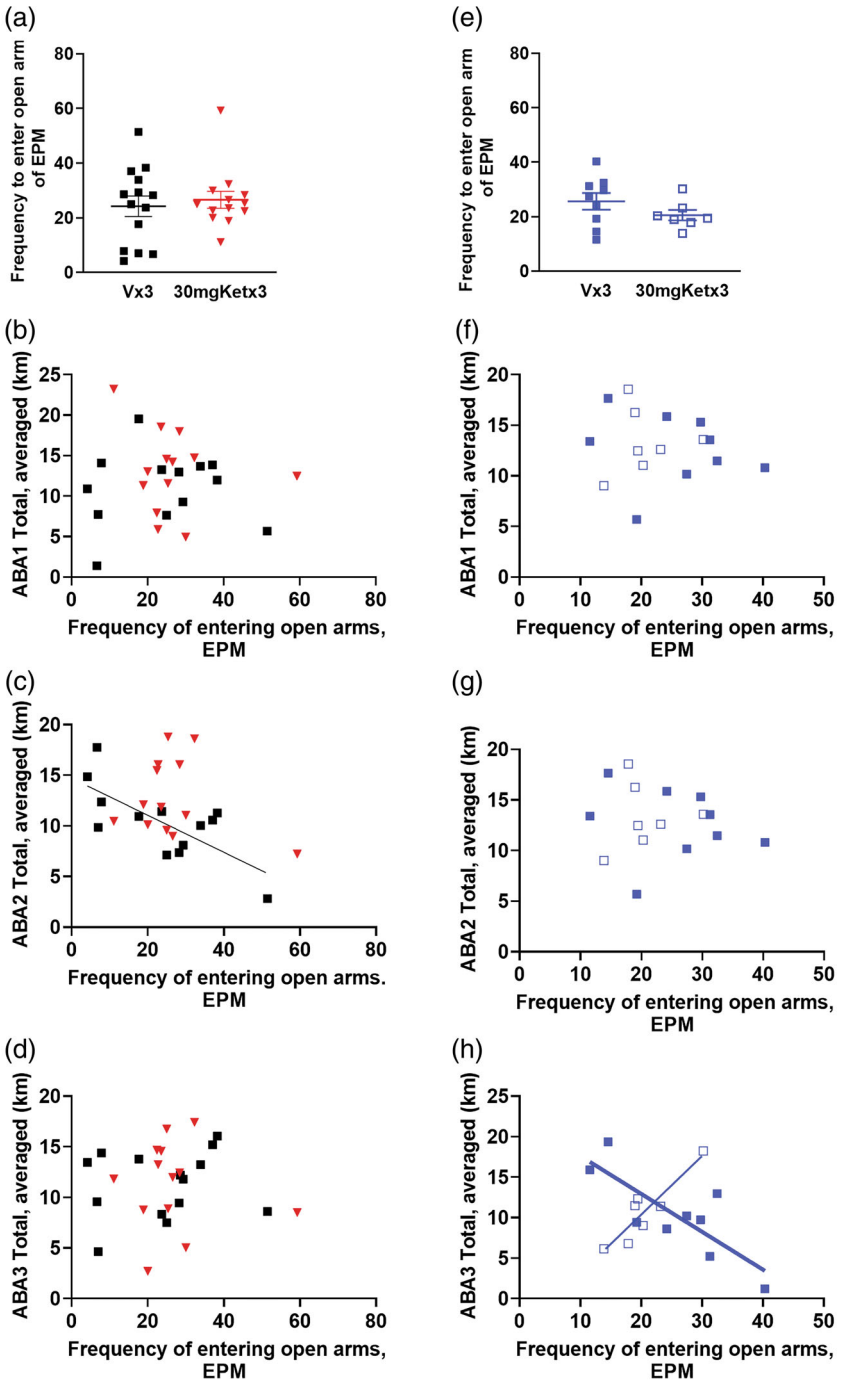


**FIGURE 5** Anxiety-like behavior of females and males as assessed by elevated plus maze (EPM) correlates negatively with wheel running for the Vx3 group and is altered by 30mgKetx3. Panels a and e: The mean frequency of entry into the open arms of the EPM show no difference across the Vx3 and 30mgKetx3 groups of females (panel a) or males (panel e). For females, $p = .6335$, $t = .4827$, mean ± SEM 24.2 ± 3.8% for Vx3; 26.6 ± 3.1% for 30mgKetx3. For males, $p = .2114$, $t = 1.309$, 25.6 ± 3.1% and $N = 9$ for Vx3; 20.5 ± 1.9% and $N = 7$ for 30mgKetx3. Panels b, c, and d: Pearson's correlations between females' frequency of entry into the open arm and total running during the days of FR of ABA1 (panel b), ABA2 (panel c), and ABA3 (panel d). The correlation was significant for the Vx3 group of females during ABA2 only ($R = -.7237$, $p = .0052$, $N = 13$ for Vx3; $R = -.2225$, $p = .4649$, $N = 14$ for 30mgKetx3) ($z = 2.61$, $p = .0091$ indicating significant difference in correlation). Panels f, g, and h: Pearson's correlations between males' frequency of entry into the open arm and total running during the days of FR of ABA1 (panel f), ABA2 (panel g), and ABA3 (panel h). The correlations were significant during ABA3 and oppositely for the two treatment groups ($p = .0099$, $R = -.7965$, $N = 9$ for Vx3; $p = .0047$, $R = .9081$, $N = 7$ for 30mgKetx3; $z = -4.04$, $p = .0001$, comparing correlations of the two groups). The correlations were not significant during ABA1 for the Vx3 ($p = .6285$ and $R = -.1878$ for Vx3) or the 30mgKetx3 group ($p = .7874$ and $R = .1263$), nor during ABA2 for the Vx3 ($p = .3780$, $R = -.3351$) or 30mgKetx3 ($p = .5585$, $R = -.2698$) groups.

explored the ability of ketamine to reduce anorexia-like maladaptive behaviors in an animal model of anorexia nervosa in adulthood.

Ketamine has been welcomed by many clinicians as one of the most impactful new drugs for treating depression: it is more rapid in onset and more enduring in its efficacy (Monteggia & Zarate Jr., 2015). As for anorexia nervosa, some studies have alluded to the potential utility of ketamine (Mills et al., 1998). Our earlier pre-clinical study indicating the long-lasting efficacy of a single injection of ketamine to female mice undergoing ABA during mid-adolescence (Chen, Sherpa, & Aoki, 2018) served as a clue for exploring ways to treat anorexia nervosa with ketamine (Calabrese, 2022; Scolnick et al., 2020). However, the finding that ketamine could exacerbate ABA vulnerability of mice when delayed to ABA2 in late adolescence (Aoki, 2020) was disconcerting and disappointing. In the current study, we show that three daily sub-anesthetic doses of ketamine, instead of a single injection, in late adolescence have three ameliorative effects upon adult female mice. Acutely, ketamine improves animals' ability to suppress wheel running during hours of food availability (Figure 3b, e; Table 3b). With delay, into adulthood, it also improves animals' ability to suppress wheel running during the hours leading up to feeding (Figure 3h, j; Table 3b). Acutely (Figure 4b) and over a longer term spanning into adulthood (Figure 4c, d), it enables animals to

compensate better for weight loss associated with excessive exercise, resulting in improved weight retention. Ketamine's ameliorative effects were also seen in adult male mice but only weakly. These findings support the notion that multiple sub-anesthetic doses of ketamine may be helpful in reducing relapse among adult females with anorexia nervosa but that one needs to be mindful of potential sex- and age-related differences in the action of ketamine.

Our measurement of FAA was the sum of wheel running during the 6 h preceding the feeding period, corresponding to the last 6 h of the light phase. Animals without the imposition of FR rarely run during these hours (Chowdhury et al., 2013). This means that ABA disrupts the sleep–wake cycle. What impact this disruption has on energy metabolism and neuromodulators is an important question for future studies.

### 4.1 | Ketamine's efficacy revealed through correlation analyses

We noted dramatic changes brought on by ketamine, revealed in the relationship between weight and FAA (Figure 4). These correlational analyses (in addition to the assessment of group mean differences and within-group analyses that follow each subject's progress through repeated ABA) provided further revelation about the drug's efficacy by accounting for a *spectrum* of individual differences in ABA vulnerability prior to ketamine administration.

Among animals without ketamine treatment, two maladaptive traits correlated—FAA and restricted food consumption (Figure 4). The correlation indicated that those that were hyperactive were the same individuals that failed to gain body weight during the subsequent feeding period. This is the paradoxical but core maladaptive behavioral trait of individuals diagnosed with anorexia nervosa (Beadle et al., 2015; Beumont et al., 1994; Carrera et al., 2012; Davis et al., 1999; Kron et al., 1978). Conversely, those individuals that were relatively less hyperactive were the same individuals that were better at gaining body weight during the subsequent feeding period. This strong correlation could be due to the two symptoms stemming from a common source –namely, heightened anxiety (Dellava et al., 2010; Kaye et al., 2004). Remarkably, the relationship between FAA and weight *reversed* for the 30mgKetx3 group: the individuals that ran more gained more weight during the subsequent hours of food availability (Figure 4), indicating that animals had switched their behavior from the paradoxically maladaptive one to an adaptive one. With this switch, animals that ran more compensated for the excessive energy expenditure by increasing food consumption and/or running less during the hours of food accessibility.

This interpretation of data is supported by a recent article (Cichon et al., 2023; Li et al., 2023) suggesting that ketamine works through a global shift in neural pathways—blocking those that had been active (which, for our mice, were the pathways linking the two maladaptive traits–running and reduced feeding). By blocking the pathways underlying maladaptive behaviors, an alternative set of pathways could begin to dominate behavior (for our ABA mice, food consumption and suppression of wheel running). This finding parallels the report of treatment-resistant depressed subjects treated with ketamine: authors report that subjects' "belief-updating became more optimistically biased" following a single infusion with ketamine, thereby enabling patients to escape the persistently depressed state (Bottemanne et al., 2022).

### 4.2 | The putative pathways affected by the sub-anesthetic dose of ketamine

Although research using animals preclude probing into animals' beliefs or emotion (LeDoux, 2012), we can surmise ABA animals' motivation to run, based on the strong correlation between behavior on the EPM and running. EPM is widely used to assess anxiety-like behavior of rodents, based on the interpretation that innate anxiety of being found by a predator in the open arms promotes the tendency of animals to remain in the more protected closed arms of the EPM. Conversely, the motivation to explore would promote animals to investigate the open arms as well, overriding their innate anxiety of being preyed upon (Chowdhury et al., 2021; Pellow & File, 1986). We confirmed earlier findings that Vx3 females' running correlates with anxiety-like behavior on the EPM, based on the significantly negative correlation of the two variables—animals that run more exhibit stronger anxiety-like behavior of remaining in the closed arms of the EPM. This correlation is observed for EPM tested in the midst of ABA1 or ABA2 or after weight restoration from ABA (Aoki & Santiago, 2022; Wable, Min, et al., 2015; and present findings). Had the correlation been positive, our interpretation would have been that wheel running is motivated more strongly by exploratory behavior. One remarkable effect of 30mgKetx3 for females was a loss of this correlation between anxiety-like behavior on the EPM and running during ABA2. We also observed the loss of correlation with maturation—during ABA3. These disappearances of correlation suggest that anxiety ceased to be a significant single factor influencing wheel running. One additional factor contributing to wheel running is habitual behavior that is insensitive to action-outcome contingency. The cortico-striatal pathway is known to dictate this behavior (Balleine et al., 2007). Indeed, our earlier finding indicates that chemogenetic manipulation of this cortico-striatal pathway can modulate FAA of animals experiencing ABA2 (Santiago et al., 2021). Wheel running enhances the release of dopamine in striatum (Bastioli et al., 2022). The mechanism underlying regulation of dopamine release may have been altered by maturation and/or by 30mgKetx3. Thirdly, the motivation to explore during ABA3 may have begun to contribute more towards wheel running, perhaps with maturation of the hippocampus (Buzsaki & Moser, 2013), known to be slower in maturation, relative to sensory cortices (reviewed in Aoki & Santiago, 2022). Our previously published data indicate that one contributor to the neurobiological substrate linking anxiety-like behavior to ABA running may be the $\alpha4\beta\delta$-$GABA_A$ receptors expressed by non-pyramidal neurons in the dorsal hippocampus, since knock-down of $\alpha4$ subunit of $GABA_A$ receptors in these neurons causes loss of this correlation (Supplemental Material in Aoki & Santiago, 2022).

**TABLE 4** One-way repeated measures ANOVA results comparing BW and wheel activity across the three ABA periods of the 30mgKetx3 male group (Table 4a) and Vx3 male group (Table 4b).

a. ANOVA comparisons of male Vx3's BW and wheel activity across ABA periods ($N = 9$)

| | Mean 1 | Mean 2 | *p*-value | Mean difference | 95% CI |
|---|---|---|---|---|---|
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA2 | 0.7890 ± 0.008951 | 0.8342 ± 0.005607 | .0021 | −0.04529 ± 0.008697 | −0.07014 to −0.02044 |
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA3 | 0.7890 ± 0.008951 | 0.8466 ± 0.006647 | .0005 | −0.05763 ± 0.008914 | −0.08310 to −0.03216 |
| BW FR3 @ 7 p.m., relative to baseline: ABA2 vs. ABA3 | 0.8342 ± 0.005607 | 0.8466 ± 0.006647 | .1038 | −0.01234 ± 0.005227 | −0.02727 to 0.002597 |
| FA increase (Avg of FR)–(Avg of baseline): ABA1 vs. ABA2 | −1.581 ± 0.2198 | −1.825 ± 0.2996 | .7839 | 0.2446 ± 0.3623 | −0.7906 to 1.280 |
| FA increase (Avg of FR)–(Avg of baseline): ABA1 vs. ABA3 | −1.581 ± 0.2198 | −1.505 ± 0.2915 | .9717 | −0.07572 ± 0.3313 | −1.022 to 0.8709 |
| FA increase (Avg of FR)–(Avg of baseline): ABA2 vs. ABA3 | −1.825 ± 0.2996 | −1.505 ± 0.2915 | .6892 | −0.3203 ± 0.3804 | −1.407 to 0.7668 |
| FAA increase (Avg of FR)–(Avg of baseline): ABA1 vs. ABA2 | 1.799 ± 0.4031 | 0.9609 ± 0.4444 | .5319 | 0.8377 ± 0.7511 | −1.309 to 2.984 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA3 | 1.799 ± 0.4031 | 0.9936 ± 0.4419 | .5504 | 0.8050 ± 0.7438 | −1.320 to 2.930 |
| FAA increase (Avg of FR)–(Avg of baseline): ABA2 vs. ABA3 | 0.9609 ± 0.4444 | 0.9936 ± 0.4419 | .9982 | −0.03269 ± 0.5789 | −1.687 to 1.621 |

b. b. ANOVAs comparisons of male 30mgKetx3's BW and wheel activity across ABA periods ($N = 8$)

| | Mean 1 | Mean 2 | *p*-value | Mean difference | 95% CI |
|---|---|---|---|---|---|
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA2 | 0.7627 ± 0.01605 | 0.8195 ± 0.009080 | .0095 | −0.05686 ± 0.01344 | −0.09645 to −0.01728 |
| BW FR3 @ 7 p.m., relative to baseline: ABA1 vs. ABA3 | 0.7627 ± 0.01605 | 0.8377 ± 0.005615 | .0024 | −0.07505 ± 0.01382 | −0.1157 to −0.03436 |
| BW FR3 @ 7 p.m., relative to baseline: ABA2 vs. ABA3 | 0.8195 ± 0.009080 | 0.8377 ± 0.005615 | .0540 | −0.01819 ± 0.006299 | −0.03674 to 0.0003597 |
| FA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA2 | −1.440 ± 0.2799 | −2.313 ± 0.2993 | .0334 | 0.8732 ± 0.2692 | 0.08032 to 1.666 |
| FA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA3 | −1.440 ± 0.2799 | −1.512 ± 0.3326 | .9540 | 0.07232 ± 0.2466 | −0.6539 to 0.7985 |
| FA increase (Avg of FR)-(Avg of baseline): ABA2 vs. ABA3 | −2.313 ± 0.2993 | −1.512 ± 0.3326 | .0123 | −0.8009 ± 0.1993 | −1.388 to −0.2140 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA2 | 3.551 ± 0.6442 | 2.112 ± 0.6322 | .2181 | 1.439 ± 0.7713 | −0.8324 to 3.711 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA1 vs. ABA3 | 3.551 ± 0.6442 | 1.249 ± 0.4490 | .0192 | 2.302 ± 0.6276 | 0.4535 to 4.150 |
| FAA increase (Avg of FR)-(Avg of baseline): ABA2 vs. ABA3 | 2.112 ± 0.6322 | 1.249 ± 0.4490 | .2767 | 0.8628 ± 0.5129 | −0.6476 to 2.373 |

*Note*: All values are shown as mean ± SEM. One-way repeated measures ANOVAs were used to compare the three time points. Data from FR1 was excluded from averaging since animals are not yet experiencing FR-evoked hunger on FR1. ABA1 had 3 days of FR (FR1, FR2, FR3), from which body weight (BW) and running data during FR2 and FR3 were averaged. ABA2 and ABA3 had 4 days of FR (FR1, FR2, FR3, FR4), from which BW and running data during the latter 3 days were averaged. Baseline BW and running were assessed during the 2 days preceding FR, then averaged and subtracted from the FR averaged values. Running activity was binned as follows: FAA = food anticipatory activity, from 1 p.m. to 7 p.m.; FA = during the 2 h of food availability, from 7 p.m. to 9 p.m. Wheel activity is expressed as KM. *P*-values highlighted in red are significant (<.05).

This study was the first, to our knowledge, to examine EPM behavior of male mice that had undergone ABA. Two unexpected findings were made: (1) the negative correlation between EPM behavior and wheel running emerged during ABA3 among the Vx3 group, rather than during ABA2, which is the time that correlation was observed for females; and (2) 30mgKetx3 reversed the correlation from negative to positive during ABA3. The first finding suggests that maturation of the neural circuit associating anxiety-like behavior to

wheel running may be delayed for males, relative to females. The second finding suggests that the single most influential factor promoting wheel running of males switches from anxiety to exploration, following 30mgKetx3 treatment.

### 4.3 | Putative mechanisms underlying the 30mgKetx3's reduction of ABA vulnerability

One dominant view regarding the mechanism of action of ketamine as an antidepressant is that sub-anesthetic doses of ketamine binds selectively to synapses within highly active synaptic pathways of prefrontal cortex (Luscher et al., 2020; Miller et al., 2014; Miller et al., 2017), amygdala (Luscher et al., 2020), hippocampus (Carreno et al., 2016) and lateral habenula (Yang et al., 2018). This is because ketamine only binds to NMDA receptors in the open state. The efficacy of ketamine as an antidepressant becomes manifest in animal models of depression *only after depression has been induced experimentally* and after key populations of neurons modulating mood enter a heightened state of activity, making those hyperactive neurons that underlie maladaptive behavior more receptive to ketamine blockade (Luscher et al., 2020; Miller et al., 2014; Yang et al., 2018).

Using this same reasoning, we surmise that the acute effect of 30mgKetx3 in female ABA brains is to also dampen the overactive excitatory synapses underlying ABA's maladaptive behavior of excessive running. One prediction from this hypothesis is that the sub-anesthetic dose of ketamine must be administered after animals have begun to exhibit ABA behavior. These reports cited above were not accompanied by findings of the effect of sub-anesthetic doses of ketamine during non-depressed states. On the other hand, there are reports indicating that ketamine could be a prophylactic agent to chronic-stress induced depression (Dolzani et al., 2018) (rev. by Evers et al., 2022). It would be interesting to assess anxiety-like behavior of ABA mice at additional time points and to determine whether sub-anesthetic doses of ketamine work as a prophylactic agent to ABA vulnerability when administered during recovery phases.

### 4.4 | Conclusion, limitations of the current study and future directions

An earlier finding that served as a foundation for this study was that ketamine treatment during ABA1 in mid-adolescence ameliorates subsequent ABA vulnerability (Chen, Sherpa, & Aoki, 2018). Because knowledge about the risk factors of treating developing brains with ketamine is limited (Wolfson et al., 2023), translation of this finding to treatments of teenagers experiencing their first onset of anorexia nervosa would be difficult to recommend. In contrast to this earlier finding, the present exploratory study shows promise that maladaptive behaviors associated with repeated ABA in adult mice and therefore also relapse of anorexia nervosa of humans may be suppressed by ketamine treatments. Our study is exploratory, because it requires replication by other labs and ours, focused on fewer parameters in the analysis. The putative sex differences revealed in the current study (Supplemental Material) highlight the importance of developing sex-specific recommendations for treating anorexia nervosa and additional studies that focus on sex-differences in the action of ketamine in ameliorating anorexia-like maladaptive behaviors. More studies with animals are needed to find dosing schedules, including the timing of ketamine injections that (1) reduce weight loss more rapidly for females (e.g., during ABA2, rather than in ABA3), perhaps by aiming to reduce post-prandial and FAA running; (2) reduce wheel activity and improve weight retention more effectively for males (Supplementary Figures 2 and 3). Future studies that assess synapses that are altered stably by ketamine may reveal pathways underlying maladaptive versus adaptive behaviors. The potential benefit of co-administering ketogenic diet with ketamine (Calabrese et al., 2022) would also be worthy of study.

## AUTHOR CONTRIBUTIONS

**Sebastian Goodwin-Groen:** Data curation; formal analysis; funding acquisition; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Yiru Dong:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; 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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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results.

## 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 at the end of this article.

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