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Effects of Selective Breeding, Voluntary Exercise, and Sex on Endocannabinoid Levels in the Mouse Small-Intestinal Epithelium

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ABSTRACT

The endocannabinoid (eCB) system in the gut communicates with the body and brain as part of the homeostatic mechanisms that affect energy balance. Although perhaps best known for its effects on energy intake, the eCB system also regulates voluntary locomotor behavior. Here, we examined gut eCB concentrations in relation to voluntary exercise, specifically in mice selectively bred for high voluntary wheel running behavior. We measured gut eCBs in four replicate non-selected Control (C) lines and four replicate lines of High Runner (HR) mice that had been selectively bred for 74 generations based on the average number of wheel revolutions on days 5 and 6 of a 6-day period of wheel access when young adults. On average, mice from HR lines run voluntarily on wheels ~3-fold more than C mice on a daily basis. A recent study showed that circulating levels of primary endocannabinoids 2-arachidonoyl-*sn*-glycerol (2-AG) and anandamide (AEA) are altered by six days of wheel access, by acute wheel running, and differ between HR and C mice in sex-specific ways [1]. We hypothesized that eCBs in the upper small-intestinal epithelium (i.e., proximal jejunum), a region firmly implicated in eCB signaling, would differ between HR and C mice (linetype), between the sexes, between mice housed with vs. without wheels for six days, and would covary with amounts of acute running and/or home-cage activity (during the previous 30 minutes). We used the same 192 mice as in [1] , half males and half females, half HR and half C (all 8 lines), and half either given or not given access to wheels for six days. We assessed the eCBs, 2-AG and AEA, and their analogs docosahexaenoylglycerol (DHG), docosahexaenoylethanolamide (DHEA), and oleoylethanolamide (OEA). Both 2-AG and DHG showed a significant 3-way interaction of linetype, wheel access, and sex. In addition, HR mice had lower concentrations of 2-AG in the small-intestinal epithelium when compared to C mice, which may be functionally related to differences in locomotor activity or to differences in body composition and/or food consumption. Moreover, the amount of home-cage activity during the prior 30 min was a negative predictor of 2-AG and AEA concentrations in jejunum mucosa, particularly in the mice with no wheel access. Lastly, 2-AG, but not AEA, was significantly correlated with 2-AG in plasma in the same mice.

1. Introduction

Endocannabinoids (eCBs) are lipid-derived signaling molecules that bind and activate G-protein-coupled cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors found throughout the body. The eCBs, their receptors, and their biosynthetic and degradative enzymes – collectively termed the eCB system – play an integral role in homeostasis, including the control of energy balance, body composition, and appetite regulation and food intake, through mechanisms that include indirect and direct control of afferent vagus nerve signals to the brain from the gastrointestinal tract [2–7]. Notably, a variety of studies suggest that overactive eCB signaling contributes to diet-induced obesity [2,8–12], which precedes and impacts metabolic conditions, cardiovascular disease, diabetes, and increased risk of cancer(s) and infection from communicable diseases, including COVID-19 [13,14]. More specifically, the proximal small intestine, in contrast to the brain and other peripheral locations, is

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implicated as a key regulatory region for eCB control of feeding behavior and resultant body composition [see for example, 5,7,15,16].

On the other hand, energy expended through physical activity and voluntary exercise are important components of the overall energy budget in both humans and rodents [17], and the eCB system has also been linked to both physical activity and exercise [1,18–25]. Given the eCB system's role in exercise and energy homeostasis, further research aimed at identifying relationships between gut-brain eCB signaling and exercise is needed. The present study is the first to measure gut eCB content in the context of voluntary exercise behavior and artificial selection for voluntary exercise. We aimed to evaluate levels of the primary eCBs, the monoacylglycerol 2-arachidonoyl-*sn*-glycerol (2-AG), and the fatty acid ethanolamide, arachidonoyl ethanolamide (AEA, anandamide), as well as their lipid analogs docosahexaenoylglycerol (DHG), docosahexaenoylethanolamide (DHEA), and oleoylethanolamide (OEA) in the upper small-intestinal epithelium of mice from lines selectively bred for high voluntary wheel-running behavior (High Runner; HR mice) as compared with those from non-selected Control (C) lines [1,26]. We also quantified sex differences and the effects of six days of opportunity to run on wheels.

The five analytes we examined have varying functions relevant to the gut-brain axis (e.g., appetite, reward) and control of voluntary locomotor behavior. Compared to other lipid-derived signaling molecules, more is known about the endocannabinoids 2-AG and AEA, the primary subjects within the existing body of eCB research. However, in the present study, we also quantified two other fatty acid ethanolamides, OEA and DHEA. In contrast to appetite-stimulating properties of AEA, OEA promotes satiety and fat catabolism, and suppression of food intake [27–30]. DHEA is lesser studied; however, it has been reported to be involved in glucose balance and CB_1 receptor expression in myoblasts [31], and modulating cytokine release of interleukin-6 in the periphery [32]. In addition, we measured levels of the monoacylglycerol, DHG, an ω-3 analog of 2-AG (an ω-6 eCB), which has anti-inflammatory properties [33,34].

Three studies in the selectively bred HR lines of mice have examined the eCB system. When systemically injected with the CB_1 antagonist rimonabant, HR females decreased running more than C females over the following hour, while males showed no linetype difference [19]. Moreover, treatment of both female and male HR mice with the CB_1 agonist, WIN 55,212-2, led to decreased running when compared to C mice [20]. Thompson et al. [1] measured plasma 2-AG and AEA in HR and C mice of both sexes, with or without wheel access for six days (we collected upper small-intestinal epithelium from the same individual mice). In brief, females had lower plasma 2-AG than males, with a main effect of sex but not linetype. 2-AG was also lower in mice that received wheel access, except in C males [1]. Additionally, the amount of prior running during the previous 30 min before plasma collection was not a significant predictor of 2-AG concentrations. Females had higher levels of AEA than males, and there was an interaction between wheel access and linetype indicating that wheel access increased AEA in C mice, while decreasing AEA in HR males. In contrast to 2-AG, the amount of prior running was a positive predictor and home-cage activity was a negative predictor of plasma AEA concentrations. Based on these previous studies of HR mice [1,19,20], we expected sex effects and possible interactions of linetype, sex, and wheel access for jejunal 2-AG and AEA, but analogs DHG, DHEA, and OEA were novel targets of study for the HR mice.

2. Materials & procedures

2.1. Ethical approval

All experimental procedures were approved by the UC Riverside Institutional Animal Care and Use Committee.

2.2. Selection experiment

Outbred Hsd:ICR mice (*Mus domesticus*) were obtained from Harlan Sprague Dawley (Indianapolis, Indiana, USA) and randomly separated into eight closed lines for a long-term artificial selection experiment that was begun in 1993 [26]. Four lines became the selected High Runner (HR) linetype and the other four were designated as Control (C) lines. At sexual maturity, all mice are provided access to Wahman-type running wheels (1.12 m circumference) for six days, with wheel-running revolutions recorded in 1-min bins for ~23 hours per day (1 hour used to download data and check mice). Mice in the HR lines are chosen to breed based on their average number of wheel revolutions on days five and six of this period, and within-family selection is used [26]. By approximately generation 20, HR lines reached selection limits at which they ran ~2.5-3-fold more revolutions per day as C mice on average [35–37].

2.3. Tissue sample collection

Adult mice (n = 192, sampled from all 8 lines) from generation 74, half HR and half C, half male and half female, were either allowed access to wheels for six days or kept without access to wheels. All mice were fed a standard diet (Teklad Rodent Diet W-8604, 14% kJ from fat, 54% kJ from carbohydrates, and 32% kJ from protein, no added sugars [less than ~9% naturally occurring sugars by weight, mostly from grains]). Wheel revolutions for mice with wheels were recorded for 23 hours per day, and home-cage activity was recorded for all mice for ~23 hours per day ([1]) for wheel and activity data). We did not choose to provide locked wheels for the mice without wheel access, as HR mice climb more than C mice when given locked wheels [38]. On day 6 animals were anesthetized with isoflurane, and blood plasma [1] and jejunum mucosa scrapings were preserved. Specifically, jejunum was rapidly collected, washed with ice-cold phosphate-buffered saline (PBS) on ice, sliced longitudinally, scraped with a glass slide to obtain mucosa, then frozen in liquid nitrogen. Animals were on a reversed photoperiod, with lights off from 0700 h to 1900 h, so that sampling could occur during the time of peak wheel running. Sampling occurred from ~0900 h to 1300 h (from 2-6 hours after lights off). Mice were 71-91 days old at the time of sampling.

2.4. Ultra-performance liquid chromatography/tandem mass spectrometry

Frozen jejunum mucosa samples were weighed and subsequently homogenized in 1.0 mL of methanol solution containing internal standards (d4-FAEs, d5-2-AG 0.26 mM, 19:2 DAG). Lipids were extracted with chloroform (2.0 mL) and washed with 0.9% saline (0.9 mL). Organic phases were collected and fractionated by open-bed silica gel column chromatography as previously described [15]. Eluted fractions were dried under N^2 and reconstituted in 0.1 mL of methanol:chloroform (9:1) for liquid chromatography/tandem mass spectrometry analyses. Lipids were analyzed using a Waters Acquity I-Class Ultra Performance Liquid Chromatography system coupled to a Waters TQS-micro Triple Quadrupole Mass Spectrometer as previously described [39]. Lipids were separated using an Acquity UPLC BEH C18 column (50 × 2.1 mm; i.d. 1.7 μm), eluted by a gradient of methanol in water (0.25% acetic acid, 5mM ammonium acetate) (from 80 to 100% methanol in 2.5 minutes, 100% 2.5-3.0 minutes, 100-80% 3.0-3.1 minutes) at a flow rate of 0.4 mL/minute. Column temperature was kept at 40°C and samples maintained in the sample manager at 10°C. Argon was used as the collision gas.

2.5. Statistical analyses

Following previous studies using these eight lines of mice [e.g., 1,26, 40], jejunum endocannabinoid concentrations were analyzed by nested analysis of variance (SAS Procedure Mixed). Line nested within linetype

(HR vs. C) was a random effect and we used covariates of age (mice were 71-91 days old) and the time of day that tissue sampling occurred. Another factor used in the current analyses was mini-muscle status (determined at dissection). The mini-muscle phenotype is caused by a recessive allele that, when homozygous, reduces triceps surae and total hindlimb muscle mass by ~50% and has pleiotropic effects on numerous other traits [41,42]. We ran this analysis for all five lipids (2-AG, AEA, DHG, DHEA, and OEA) using all mice (n = 183-190), mice with wheel access (n = 90-92), and mice without wheel access (n = 91-95).

Following Thompson et al. [1], we repeated the preceding analyses with physical activity covariates: amount of wheel running (revolutions/unit time) and home-cage activity (HCA) in the 30 minutes prior to sampling. For the analysis of all mice, we used both covariates and assigned running values of zero to mice without wheel access. Then we performed separate analyses for the mice with wheels using both running and HCA covariates, and for the mice without wheel access using only the HCA covariate. We used 30 minutes of activity for consistency with Thompson et al. [1], who computed the number of wheel revolutions in each minute before plasma and tissue sampling, from 1 to 10 min before, and then in 10-min bins from 10 to 120 min before sampling. After examining models using each of these alternative covariates, they determined that 30 minutes provided the best fit. In the Thompson et al. [1] analysis, it was established that these physical activity covariates could be significant predictors of plasma endocannabinoid concentrations.

Dependent variables were transformed when needed to improve the normality of residuals. Residuals that were >3 standard deviations above or below the mean were excluded from analyses. Main effects were considered statistically significant when $P \leq 0.05$. Following Thompson et al. [1], interactions of main effects were considered significant when $P \leq 0.10$ because the power to detect interactions is generally substantially lower than for detecting main effects in ANOVAs [43,44]. Least squares means and associated standard errors from SAS Procedure Mixed were inspected to determine the directions of main effects and interactions. In addition, for some pairwise comparisons of subgroup means, we refer to differences of least squares means from SAS Procedure Mixed.

A total of ~450 P values are presented in the present and supplemental analyses, representing all of the primary results (excluding time of day, which was a nuisance variable). Based on the positive False Discovery Rate (pFDR) procedure as implemented in SAS Procedure Multtest, an appropriate cutoff would be ~P = 0.007 to control the false discovery rate at 0.05. However, simulations to explore statistical power indicate generally deflated Type I error rates for linetype comparisons in this selection experiment for $\alpha = 0.05$ [45]. Thus, for simplicity, all P values reported in the text are the nominal ones, not adjusted for multiple comparisons.

3. Results

Of the five endocannabinoids analyzed in the small-intestinal epithelium in the present study, 2-AG had the highest concentrations for all mice, with group averages between 54-88 nmol/g (Fig. 1 shows transformed [raised to 0.6 power] least squares means from SAS analyses). DHG was second highest with group average concentrations falling between 8.4-13.5 nmol/g (Fig. 2; raised to 0.5 power). AEA and DHEA concentrations were the lowest, measured in the picomole range of 16.4-19.6 pmol/g (Supplemental Fig. 1: AEA $\log_{10}$-transformed data and Supplemental Fig. 2: DHEA data transformed by raising to the 0.4 power). OEA concentrations were 172-201 pmol/g (Supplemental Fig. 3; data transformed by raising to the 0.5 power). In comparison to intestinal tissue, Thompson et al. [1] reported (in the same mice) plasma AEA levels between 0.18-0.22 pmol/mL and 2-AG levels between 62-98 pmol/mL. Mini-muscle status (see Methods), age, and time of day were included as factors or covariates in all analyses. Time of day was not a statistically significant predictor for intestinal eCB concentrations in any group or analysis. Supplemental Table 1 shows a summary of all statistical analyses.

3.1. Wheel running and HCA

Wheel revolutions and home-cage activity (HCA) for Day 5 of the experiment are reported in Thompson et al. [1]. Briefly, as expected, HR mice ran significantly more than C mice ($P = 0.0004$), but neither the effect of sex ($P = 0.1431$) nor the sex-by-linetype interaction ($P = 0.1576$) were significant. For HCA, mice with wheel access had significantly reduced activity levels; the reduction was greater for HR mice than for the C mice; and females and HR mice always had higher HCA than males or C mice, respectively.

3.2. Endocannabinoid concentrations

3.2.1. Jejunum 2-AG concentrations. HR mice had lower levels of 2-AG in their jejunum mucosa than did C mice (Table 1; $P = 0.0491$) in all four experimental groups (Fig. 1). A three-way interaction among wheel access, sex, and linetype also occurred ($P = 0.0857$): levels of 2-AG were lowest for HR females with no wheel access and highest for C males with wheel access. When examining the pairwise comparisons, for these two groups the difference was significant ($P = 0.0336$). The difference between HR and C females with no wheel access was also significant ($P = 0.0376$).

Separate analyses of the mice with (n = 92) and without (n = 95) wheels (not including physical activity covariates) indicated that HR mice had significantly lower 2-AG levels in their jejunum only when they had wheel access ($P = 0.0094$ for mice with wheels, $P = 0.1720$ for

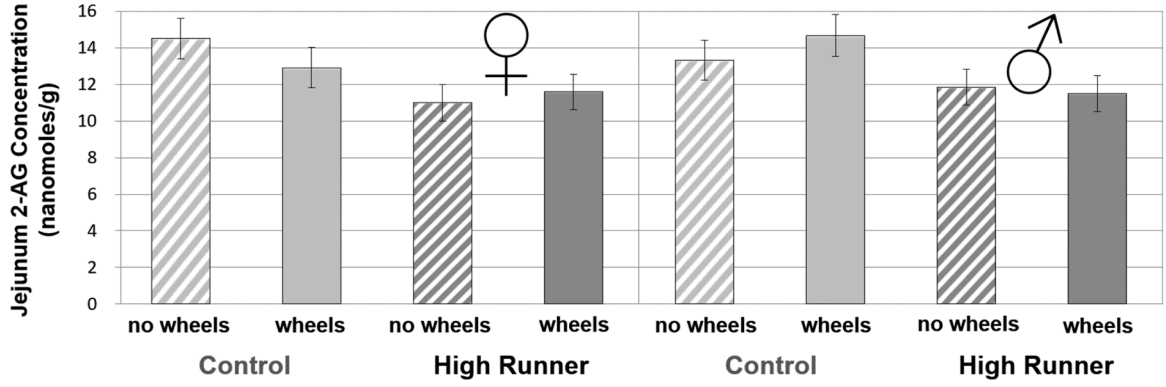

Figure 1. Concentrations of 2-AG in the jejunum mucosa collected during peak activity on the 6th night of wheel running (n = 190). Overall, 2-AG was higher in Control mice than in High Runner mice ($P = 0.0491$) and a three-way interaction among wheel access, sex, and linetype was present ($P = 0.0857$). Values are LS means ± standard errors from SAS Proc Mixed for data transformed by raising to the 0.6 power. See Table 1 for full statistical analysis.

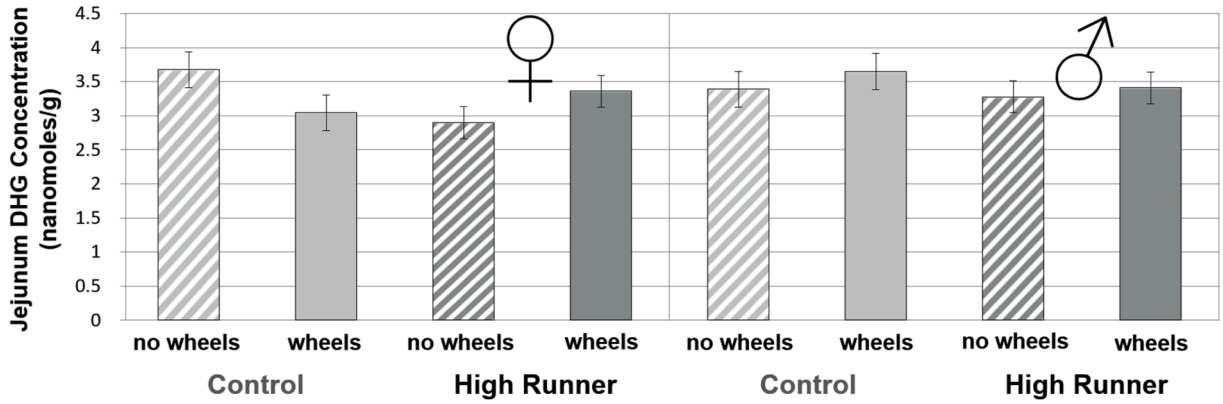

Figure 2. Concentrations of DHG in the jejunum mucosa collected during peak activity on the 6th night of wheel running (n = 190). There was a three-way interaction among wheel access, sex, and linetype (P = 0.0529). Values are LS means ± standard error from SAS Proc Mixed for data transformed by raising to the 0.5 power. See Table 1 for full statistical analysis.

Table 1
Three-way analysis of jejunum mucosa concentrations of 2-AG, AEA, DHG, DHEA, and OEA with no physical activity covariates (n = 183-190). See Fig. 1 and 2 for graphical representations of adjusted group means of 2-AG and DHG, respectively.

	2-AG *n = 190*			AEA *n = 183*			DHG *n = 190*			DHEA *n = 189*			OEA *n = 190*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Sex	1,6	0.33	0.5847	1,6	1.56	0.2580	1,6	1.5	0.2669	1,6	0.21	0.6611	1,6	4.26	0.0845
Linetype	1,6	6.05	0.0491	1,6	0.05	0.8301	1,6	1.02	0.3508	1,6	0.08	0.7824	1,6	0.11	0.7496
Wheel access	1,6	0.00	0.9997	1,6	0.75	0.4208	1,6	0.12	0.7376	1,6	5.39	0.0593	1,6	1.41	0.2799
Sex * wheel access	1,6	1.08	0.3391	1,6	0.01	0.9235	1,6	1.18	0.3189	1,6	0.35	0.5763	1,6	0.15	0.7096
Sex * linetype	1,6	0.01	0.9357	1,6	0.01	0.9245	1,6	0.03	0.8639	1,6	0.37	0.5659	1,6	0.10	0.7641
Linetype * wheel access	1,6	0.05	0.8288	1,6	1.21	0.3144	1,6	2.32	0.1786	1,6	0.05	0.8342	1,6	1.50	0.2670
Sex * linetype * wheel access	1,6	4.22	0.0857	1,6	0.71	0.4315	1,6	5.79	0.0529	1,6	0.02	0.9024	1,6	0.23	0.6466
Mini-muscle	1,148	0.15	0.6977	1,141	0.37	0.5434	1,148	0.02	0.8796	1,147	0.36	0.5504	1,148	0.24	0.6277
Age	1,148	2.34	0.1279	1,141	2.20	0.1404	1,148	2.57	0.1110	1,147	1.40	0.2394	1,148	6.63	0.0110
Time of day	1,148	0.01	0.9269	1,141	0.13	0.7238	1,148	0.39	0.5334	1,147	1.78	0.1837	1,148	0.02	0.8996

mice without wheels) (Supplemental Table 1).

Mini-muscle status, age, and time of day were not significant for 2-AG when data were analyzed without running or HCA covariates (Supplemental Table 1).

3.2.2. Jejunum 2-AG concentrations with physical activity covariates

For this analysis, the amount of wheel running and HCA in the 30 minutes prior to cardiac puncture and tissue sampling were included as covariates (raised to the 0.4 power and 30 min for consistency with Thompson et al. [1]). For the mice housed with wheel access (n = 92), neither wheel running nor home-cage activity were significant predictors of 2-AG concentrations (Supplemental Table 1). However, similar to results reported above, HR mice had significantly lower 2-AG levels in their jejunum mucosa than C mice (P = 0.0296), with no effect of sex, nor a sex-by-linetype interaction. For the mice housed without wheels (n = 95), HCA was a significant negative predictor of 2-AG levels (P = 0.0333), with no effect of linetype, sex or a sex-by-linetype interaction.

To test whether acute physical activity had a different effect than the five days of wheel access (following Thompson et al. [1] and Copes et al. [46]), an additional analysis was performed for each eCB using the activity covariates, in which we included the mice housed without wheel access but we assigned them values of zero for their wheel running. In our 2-AG analysis (n = 186), the three-way interaction reported in

Table 2
Analysis of covariance of jejunum mucosa concentrations of 2-AG, AEA, DHG, DHEA, and OEA with wheel running and HCA covariates (n = 180-187), where mice without wheel access had running values set to zero.

	2-AG n = 186			AEA n = 180			DHG n = 187			DHEA n = 186			OEA n = 187		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Sex	1,6	0.10	0.7616	1,6	0.64	0.4531	1,6	0.88	0.3842	1,6	0.39	0.5543	1,6	2.87	0.1414
Linetype	1,6	2.89	0.1398	1,6	0.06	0.8223	1,6	0.07	0.7966	1,6	0.07	0.7988	1,6	0.02	0.8919
Wheel access	1,6	0.28	0.6158	1,6	0.34	0.5805	1,6	0.48	0.5143	1,6	0.00	0.9533	1,6	0.17	0.6954
Sex * wheel access	1,6	1.13	0.3288	1,6	0.00	0.9796	1,6	1.49	0.2681	1,6	0.21	0.6617	1,6	0.12	0.7406
Sex * linetype	1,6	0.00	0.9844	1,6	0.02	0.8825	1,6	0.00	0.9874	1,6	0.27	0.6207	1,6	0.01	0.9138
Linetype * wheel access	1,6	0.22	0.6558	1,6	0.06	0.8119	1,6	2.25	0.1840	1,6	0.15	0.7106	1,6	0.86	0.3898
Sex * linetype * wheel access	1,6	5.37	0.0597	1,6	0.60	0.4689	1,6	5.64	0.0551	1,6	0.00	0.9648	1,6	0.19	0.6798
Mini-muscle	1,142	0.20	0.6546	1,136	0.30	0.5873	1,143	0.10	0.7556	1,142	0.36	0.5508	1,143	0.14	0.7100
Age	1,142	1.90	0.1699	1,136	2.46	0.1190	1,143	2.55	0.1124	1,142	1.37	0.2438	1,143	6.80	0.0101
Time of day	1,142	0.11	0.7424	1,136	0.35	0.5523	1,143	0.46	0.4981	1,142	2.03	0.1560	1,143	0.01	0.9107
Running in previous 30 min	1,142	0.16	0.6919	1,136	0.20	0.6519	1,143	1.14	0.2873	1,142	0.83	0.3635	1,143	0.05	0.8216
HCA in previous 30 min	1,142	5.46	0.0208	1,136	3.32	0.0705	1,143	1.01	0.3171	1,142	0.90	0.3453	1,143	0.82	0.3680

Table 1 (Fig. 1) remained significant (P = 0.0597; Table 2); however, except for C males, levels of 2-AG were lower in mice with wheel access. The prior 30 minutes of HCA was a significant negative predictor of jejunum 2-AG (P = 0.0208), but wheel running was not.

3.2.3. Jejunum AEA concentrations

Levels of AEA in the jejunum were roughly uniform across all groups (16.6-19.6 pmol/g), with no significant main effects or interactions (Table 1; Supplemental Fig. 1) and no effect of mini-muscle status, age, or time of day (Table 1). In the separate analyses of the mice with (n = 90) and without (n = 91) wheels (not including physical activity covariates), we found no effect of sex, linetype or their interaction (Supplemental Table 1). However, mini-muscle mice had significantly higher AEA levels than non-mini muscle mice in the wheel-access group (P = 0.0454).

3.2.4. Jejunum AEA concentrations with physical activity covariates

For mice with wheel access (n = 90), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of AEA levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Supplemental Table 1). For the mice housed without wheels (n = 91), HCA was a significant negative predictor of AEA levels (P = 0.0145), with no effect of sex, linetype or their interaction (Supplemental Table 1). For the analysis of all mice (n = 180; values of zero assigned to the mice without wheel access), we found no main effects of wheel access, sex, linetype, their interactions, or effects of covariates (Table 2), though 30 min of HCA tended to negatively predict AEA concentrations (P = 0.0705).

3.2.5. Jejunum DHG Concentrations

The 2-AG endocannabinoid analog DHG showed a three-way interaction (P = 0.0529; Table 1; Fig. 2). Levels of DHG were higher in mice with wheel access, except for C females. When examining the pairwise comparisons, results were similar to those for 2-AG: for females without wheels, HR tended to have lower DHG (P = 0.0514); and HR females without wheels had lower DHG than C males with wheels (P = 0.0609). Mini-muscle status, age, and time of day were not significant predictors of DHG concentrations when data for all mice were analyzed without running or HCA covariates (Table 1).

In separate analyses of the mice with (n = 92) and without (n = 95) wheels (not including physical activity covariates), we found no effect of sex, linetype or their interaction (Supplemental Table 1). Mini-muscled mice housed without wheels tended to have lower DHG levels than non-mini-muscled mice (P = 0.0506).

3.2.6. Jejunum DHG concentrations with physical activity covariates

For mice with wheel access (n = 92), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of DHG levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Supplemental Table 1). For the mice housed without wheels (n = 95), HCA was not a significant predictor of DHG levels, with no effect of sex, linetype or their interaction. However, mini-muscle mice had significantly lower jejunum DHG than non-mini-muscled mice (P = 0.0242).

For the analysis of all mice (n = 187; values of zero assigned to the mice without wheel access), the three-way interaction observed previously without covariates (Table 1), remained significant (P = 0.0551; Table 2), and levels of DHG were higher in mice with wheel access, except for C females. There were no other main effects or interactions of wheel access, sex, and/or linetype (Table 2). Neither wheel running nor HCA were significant predictors of jejunum DHG (Table 2).

3.2.7. Jejunum DHEA concentrations

Mice with wheel access tended to have higher levels of the AEA analog DHEA (P = 0.0593; Table 1) in all four experimental groups (Supplemental Fig. 2). In the separate analyses of mice with (n = 91) and without (n = 95) wheels (not including physical activity covariates) we found no effect of sex, linetype or their interaction (Supplemental Table 1).

3.2.8. Jejunum DHEA concentrations with physical activity covariates

For mice with wheel access (n = 91), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of DHEA levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Supplemental Table 1). For the mice housed without wheels (n = 95), HCA tended to negatively predict DHEA levels (P = 0.0683), with no effect of sex, linetype or their interaction. For the analysis of all mice (n = 186; values of zero assigned to the mice without wheel access), we found no main effects of wheel access, sex, linetype, their interactions, or effects of covariates (Table 2).

3.2.9. Jejunum OEA concentrations

Male mice tended to have higher OEA levels in their jejunum mucosa than females (P = 0.0845; Table 1; Supplemental Fig. 3). In the separate analyses of the mice with (n =94) and without (n = 92) wheels (not including physical activity covariates), we found no effect of sex, linetype or their interaction (Supplemental Table 1).

3.2.10. Jejunum OEA concentrations with physical activity covariates

For mice with wheel access (n = 92), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of OEA levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Supplemental Table 1). For mice housed without wheels (n = 94), HCA tended to be a negative predictor of OEA levels (P = 0.0552), with no effects of sex, linetype or their interaction. For the analysis of all mice (n = 187; values of zero assigned to the mice without wheel access), we found no main effects of wheel access, sex, linetype, their interactions, or effects of physical activity covariates (Table 2).

3.3. Correlations between eCBs

Following Thompson et al. [1], we examined the relationship between jejunum and circulating levels of eCBs by a variety of measures. First, we calculated Pearson correlation coefficients for the raw values for jejunum and plasma analyte concentrations in all mice (statistical outliers removed). In the jejunum, all five analytes were significantly intercorrelated (Table 3: all P < 0.01), whereas 2-AG and AEA were uncorrelated in plasma ($r = 0.083$, n = 184, P = 0.261). Between tissues, only one correlation was statistically significant (plasma 2-AG and jejunum OEA, $r = -0.176$, P = 0.016).

Second, we analyzed the correlations for jejunum analytes within each of the eight experimental subgroups (n = 21–24 per group) and found values ranging from −0.10 to +0.91, with 78 of 80 values being greater than zero, and 44 of the 78 significant at P < 0.05 (Supplemental Table 2). Jejunum 2-AG and DHG were highly correlated within all eight groups (all 2-tailed P <0.01), as were jejunum AEA and OEA (all P <0.05). HR females without wheels were the only group that showed all five analytes to be significantly correlated (all P <0.01). Of the 80 possible correlations between plasma 2-AG and AEA and jejunal values for our five analytes, only six were statistically significant (P <0.05, five negative, one positive: Supplemental Table 3).

Third, the plasma and jejunum LS means for 2-AG in the eight experimental groups, when analyzed with the 30 min of previous wheel-running and HCA as covariates, were positively correlated ($r = 0.745$, P = 0.0339; Fig. 3). When we repeated the analysis without the physical activity covariates, the relationship was weaker and not significant (figure not shown; $r = 0.633$; P = 0.0922). For AEA, the corresponding values, with and without wheel-running and HCA covariates, were not

Table 3
Pearson correlations of raw values for all mice between 2-AG, AEA, DHG, DHEA, and OEA in jejunum mucosa, as well as plasma endocannabinoids. P values are for 2-tailed tests.

Correlations			Jejunum Mucosa					Plasma	
			2-AG	AEA	DHG	DHEA	OEA	2-AG	AEA
Jejunum Mucosa	2-AG	Pearson Correlation		0.372**	0.800**	0.295**	0.417**	-0.038	0.098
		Sig. (2-tailed)		2.10E-07	1.58E-43	3.66E-05	2.09E-09	0.599	0.183
		N	190	183	190	189	190	189	185
	AEA	Pearson Correlation			0.403**	0.515**	0.684**	-0.089	0.115
		Sig. (2-tailed)			1.57E-08	1.07E-13	1.44E-26	0.234	0.126
		N		183	183	182	183	182	178
	DHG	Pearson Correlation				0.216**	0.440**	-0.081	-0.023
		Sig. (2-tailed)				0.003	2.13E-10	0.268	0.759
		N			190	189	190	189	185
	DHEA	Pearson Correlation					0.586**	-0.087	0.05
		Sig. (2-tailed)					8.72E-19	0.238	0.496
		N				189	189	188	184
	OEA	Pearson Correlation						-0.176*	0.048
		Sig. (2-tailed)						0.016	0.520
		N					190	189	185
Plasma	2-AG	Pearson Correlation							0.083
		Sig. (2-tailed)							0.261
		N						189	184
	AEA	Pearson Correlation							
		Sig. (2-tailed)							
		N							185

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

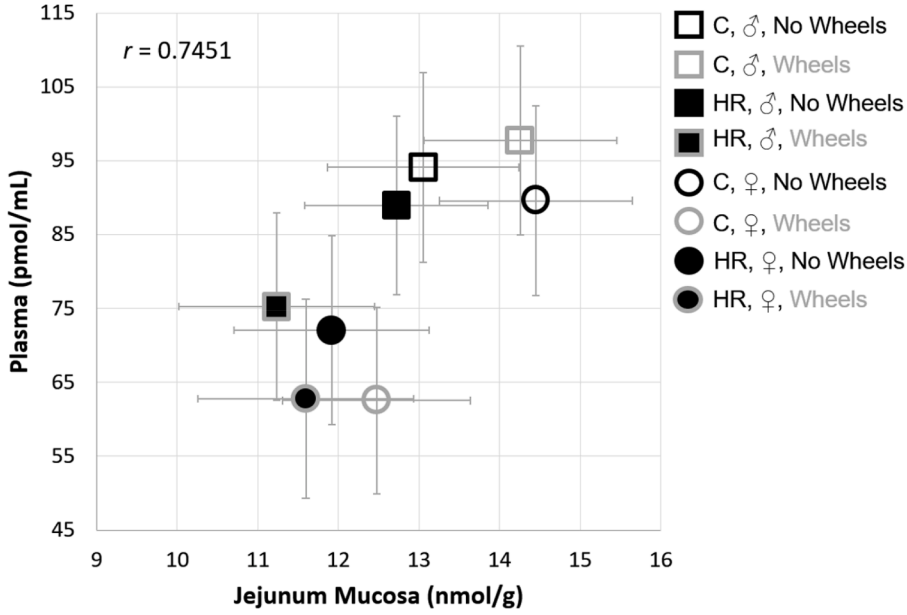

Figure 3. Correlation between group means for plasma from [1] and jejunum mucosa 2-AG. Values are LS means ± standard error from SAS Proc Mixed with the amount of wheel running and HCA in the 30 mins prior to sampling as covariates (from our Table 2 and Section 3.4 of [1]. Jejunum data was transformed by raising to the 0.6 power. The correlation is statistically significant (P = 0.0339).

correlated (figures not shown; $r = 0.284$ and P = 0.4953 with covariates; $r = 0.384$ and P = 0.3472 without covariates).

Fourth, we computed simple mean values for each of the eight subgroups (linetype by sex by wheel access; Supplemental Table 4) and found two statistically significant correlations: between jejunum AEA and DHG ($r = 0.919$, P = 0.001), and between jejunum AEA and OEA ($r = 0.721$, P = 0.044). No correlations were present between the simple subgroup means of jejunum vs. plasma eCBs.

4. Discussion

Whether genetic background, sex differences, and/or exercise modulate levels of eCBs in the gut, specifically the proximal small intestine, is unknown. We used a unique, artificial selection mouse model to test how levels of eCBs and their related lipid messengers in the small-intestinal epithelium differ between *(i)* lines of mice selectively bred for high voluntary wheel-running behavior vs. non-selected Control lines, *(ii)* males vs. females, and *(iii)* mice provided six days of wheel access vs. no wheel access. We also tested for possible interactive effects and for correlations with the amount of acute physical activity during the 30 min prior to tissue extraction. We found evidence for decreased 2-AG concentrations in the jejunum mucosa of High Runner vs. Control mice, along with three-way interactions for 2-AG and its ω-3 lipid analog DHG. In addition, the amount of home-cage activity during the prior 30

min was a negative predictor of 2-AG and AEA concentrations in intestinal epithelium, particularly in the mice with no wheel access. Furthermore, intestinal 2-AG, but not AEA, was significantly correlated with 2-AG in circulation in the same mice [1].

4.1. Previous studies of eCBs in the gut or in relation to exercise

ECBs, their biosynthetic and degradative enzymes, and their receptors have been studied in both human and rodent central and peripheral tissues. Experiments have separately examined eCB system components in the intestine or given exercise, but not together. In the intestine, several studies of diet and food intake in rodents have quantified various components of the eCB system [4,47,48], leading to the discovery that efferent vagal signals to the jejunum result in eCB production and receptor binding in the mucosal epithelium. These cannabinoids can then inhibit production of the satiety peptide cholecystokinin (CCK), thereby significantly altering the afferent gut-to-brain control of feeding [4].

One other recent study by Guida et al. [49] evaluated intestinal eCBs in a study of vitamin D deficiency and pain processing where mice received tibial and common peroneal nerve injury. In the colon, but not small intestine, vitamin D deficient mice had reduced 2-AG levels and palmitoylethanolamide (PEA) treatment elevated AEA, OEA, and DHEA in vitamin D normal mice with nerve injury. The authors interpreted their results as suggesting that vitamin D deficiency may be accompanied by increased pain and inflammation caused by reduced eCB signaling at intestinal CB1 and CB2 receptors. They also suggest that PEA may provide amelioration [49]. Future studies could examine the relationship of exercise-related pain paired with the gut eCB system.

Importantly, exercise can affect the gastrointestinal tract, digestion, and the gut-brain axis in various ways [50–53]. Exercise studies have primarily used plasma or saliva samples and they vary by acute vs. chronic paradigms and by exercise intensity and duration. Generally for humans, plasma AEA [22,24,25,54] and OEA [25] are increased following exercise, and only sometimes is 2-AG increased [24,25]. DHG and DHEA have not been previously quantified in an exercise context. We compared our results of gut eCBs to the plasma results in Thompson et al. [1] and the across-tissue similarities and differences we found are discussed below in section 4.2.

Our novel pairing of exercise and eCB measurement in the intestine limits comparisons with previous studies. Even considering "control" conditions (i.e., adults on "standard" diet, freely-fed), male mice have varying concentrations of 2-AG, AEA, DHG, DHEA, and OEA in the intestine [6,10,12,55]. Some of this variability is likely related to experimental details, such as age, the %kcal from fat in the standard diet, the use of metabolic cages that prevent coprophagia, or homogenization of whole jejunum vs. the mucosal layer. Overall, the 2-AG, AEA, and DHEA concentrations observed in the present study are within the range of values reported previously, but the group mean concentrations of DHG and OEA are lower.

4.2. Comparisons of gut and plasma endocannabinoids in this experiment and Thompson et al. (2017)

We studied the same individual mice as Thompson et al. [1] used to measure circulating endocannabinoids. Therefore, we compared 2-AG and AEA concentrations in small-intestinal epithelium and plasma by examining the statistical results reported here (Sections 3.2.1-3.3.4) with those reported in Thompson et al. [1]. In general, most of the 2-AG and AEA effects found in the proximal small intestine vs. the plasma differed. Notably, the significant main effect of sex on circulating 2-AG was absent from jejunum mucosa (Tables 1 and 2). With regard to AEA, the wheel access-by-linetype interaction, where wheel access significantly lowered plasma AEA levels in all mice but much more so in HR lines than in C lines, was not observed in the jejunum (Table 2). Additionally, the previous 30 minutes of running was a highly significant positive predictor of plasma AEA, whereas the previous amount of running did not covary with jejunal mucosa AEA (Table 2).

The one obvious similarity for jejunum and plasma [1] was the three-way interaction between linetype, sex, and wheel access for concentrations of 2-AG (our Sections 3.2.1-3.2.2 and their Sections 3.3-3.4). A three-way interaction present in both tissues and a statistically significant positive correlation between jejunum and plasma 2-AG (at the level of our eight experimental groups; Fig. 3) suggests that gut and plasma 2-AG may be regulated by common pathways and/or acting synergistically in the eCB system.

Previous research indicates a correlation of eCBs across tissues is plausible. Argueta and DiPatrizio [6] found that 2-AG and AEA concentrations are elevated in both plasma and jejunum mucosa of male C57BL/6 mice fed ad-libitum WD for 60 days when compared to their SD counterparts. Their study suggests that local signaling by eCBs in the upper small intestine, along with those in circulation, may both interact with feeding- and reward-related pathways in the brain. Thus, our 2-AG across-tissue correlation (Fig. 3) might also suggest that the periphery-to-brain communication of 2-AG may be modulated by an interaction of genetic background, biological sex, and access or no access to running wheels.

Another interesting difference between jejunum and plasma is their respective correlations between 2-AG and AEA. In the jejunum, 2-AG and AEA were considerably more positively correlated than in the plasma (Table 3). Further, besides a slight negative relation between plasma 2-AG and jejunum OEA, none of our raw analyte values were correlated with the 2-AG or AEA from Thompson et al. [1].

As our study quantified the three additional analytes DHG, DHEA, and OEA, we also tested for intercorrelations between all five lipid messengers. Overall, our analyses indicate that the raw values of all five intestinal analytes are positively related ($n = 182$-190; Table 3) but not when tested as the simple means for the eight experimental groups ($n = 8$; Supplemental Table 4). The highest correlation between the raw values of two analytes was between jejunum 2-AG and its ω-3 counterpart DHG (Table 3: $n = 190$, $r = 0.800$, $p << 0.0001$), and 2-AG and DHG were also significantly correlated within each of the eight experimental groups (Supplemental Table 2). These findings may involve the biosynthetic and/or degradative enzymes acting on both of the monoacylglycerols.

4.3. Linetype differences in relation to activity effects

In combined analyses, mice from the selectively bred HR lines had lower 2-AG in their jejunum mucosa than C mice for both sexes and housing conditions, although the magnitude of this difference varied, as evidenced by a three-way interaction (Table 1, Fig. 1). Separate analyses of mice housed with or without wheel access indicated that HR mice had lower 2-AG only with wheel access (Sections 3.2.1& 3.2.2.). Thus, the magnitude of the linetype difference varies with both sex and physical activity. A study of males found that HR mice also have greater rates of lipid oxidation than C mice during exercise at 66% of VO_2max [56], an intensity of exercise that occurs during voluntary wheel running [57]. The lipids metabolized for energy during sustained, aerobically supported exercise (as in mouse wheel running) may be drawn from various pools, including white adipose tissue [58,59] and skeletal muscle [60], in addition to digestion in the small intestine [61–63]. If the synthesis of 2-AG involves precursors that are also used during exercise-induced lipid oxidation, then perhaps jejunal 2-AG might be depleted during exercise in HR mice. However, eCBs in the intestine are likely acting locally and are produced and degraded locally. To date, we have no evidence that precursors and eCBs in the intestine may escape into circulation [e.g., see 39].

When all mice were analyzed with physical activity covariates, the prior 30 min of HCA was a significant negative predictor of 2-AG (i.e., more activity in their home-cage was associated with less 2-AG and vice versa; Table 2). In the analyses of mice without wheels, 2-AG, AEA,

DHEA, and OEA were also negatively predicted by HCA in the 30 minutes before tissue samples were extracted. Given that the eCB system is physiologically extensive and linked to many other bodily systems, these results suggest that varying levels of rodent activity in their home cages could be a confounding factor in various studies (e.g., pharmacological studies using drugs that affect locomotion) by way of small intestinal lipid messengers.

Lastly, mice with wheel access tended to have higher levels of DHEA (Table 1; Supplemental Fig. 2). DHEA is a lesser-studied fatty acid ethanolamide, and research is just beginning to elucidate its physiological role(s). For example, Kim et al. [31] treated myoblasts *in vitro* with DHEA, which resulted in higher CB_1, GLUT1, and insulin receptor mRNA expression as well as higher glucose uptake compared to controls. In addition, Meijerink et al. (2015) confirmed *in vitro* that DHEA modulates release of cytokine interleukin 6 (IL-6) in peritoneal macrophages after LPS stimulation. These findings suggest possible mechanisms (e.g., glucose metabolism, or tissue inflammation) that could connect wheel running to DHEA levels.

4.4. Sex differences in small intestine eCBs

Considering only the control groups in the present study (i.e., mice from either genetic linetype housed without wheels), we found no statistically significant sex differences for levels of eCBs and other related lipids included in our analysis (based on differences of LS Means from SAS Proc Mixed) (results not shown). However, in combined analyses of all mice, we did find evidence for interactive effects that included sex for both 2-AG and DHG (Tables 1 and 2).

To our knowledge, only one previous study of small-intestinal eCB concentrations in mice included both sexes. Perez and DiPatrizio [12] also measured 2-AG, AEA, DHG, DHEA, and OEA in small-intestinal epithelium (jejunum mucosa) of offspring from dams fed a standard (SD) or a Western-style diet (WD, high fat and sucrose), but did not compare the sexes. Based on their Table 2, female offspring from SD dams had higher concentrations of all five analytes in small-intestinal epithelium than male SD offspring. The same pattern was apparent for offspring from WD dams, with the exception of AEA. However, t-tests indicate that female offspring had significantly higher mucosal 2-AG than the male offspring from the WD dams ($t = 2.836$; df = 11, P = 0.0162), and a similar trend was present for 2-AG in offspring from SD dams ($t = 1.9340$; df = 14; P = 0.0736). Another sex-difference they report (their page 7) comes from 2-AG and AEA concentrations measured in the dams during their pre-gestation phase (i.e., adult female mice on SD or WD for 10 weeks). When compared to control mice maintained on SD (i.e., low-fat/no sucrose), female WD mice had reduced levels of 2-AG and increased levels of AEA in plasma; however, no differences were detected in levels of 2-AG or AEA in the upper small-intestinal epithelium. This result is in contrast to male mice maintained on WD for 60 days ([6]), which had elevated levels of 2-AG and AEA in both upper small-intestinal epithelium and plasma, when compared to mice maintained on SD control. The underlying molecular mechanisms for these sex-dependent discrepancies are unknown and their elucidation will be important for future investigations.

4.5. Future directions

With the addition of our study, research has found that sex differences, exercise, and genetic background may, together or on their own, modify the eCB system. Bidirectional gut-brain communication is a key component of the eCB system of particular relevance [5]. Our results may have implications for the gut-brain axis, particularly the link between proximal small intestine, midbrain, and dorsal striatum [64], brain regions critical for motivated behaviors [65,66]. The interaction between voluntary exercise and the gut-brain axis may indeed be modulated by the eCB system and/or its control of dopamine function [67], but further research is required.

Different components of the eCB system, including enzymes and cannabinoid receptors, are feasibly causing, and responding, to varying concentrations of gut eCBs. For example, lower levels of 2-AG observed in the small-intestinal epithelium of HR mice may be a result of increased activity of the degradative enzymes for 2-AG and other monoacylglycerols (e.g., monoacylglycerol lipase, MGL), and/or decreased activity of the biosynthetic monoacylglycerol enzyme, diacylglycerol lipase (DGL). Consequently, less available 2-AG might mean less activity at CB_1, and thus altered afferent signals to the CNS. Future studies should investigate receptor expression and binding and compare the two possible mechanisms underlying the decreased 2-AG in HR mice, i.e., a decreased rate of 2-AG synthesis (from 1-stearoyl-2-arachidonoyl-*sn*-glycerol [SAG]) via DGL or an increased rate of degradation (via MGL into arachidonic acid and glycerol).

Finally, future studies should examine how acute vs. chronic voluntary exercise may change eCB concentrations and communication in the proximal small intestine, and could also incorporate differences in diet (e.g., Western diet). Rodents fed a Western diet have significantly altered peripheral eCB system profiles and eCB endogenous activity at peripheral CB_1 receptors is crucial for driving hyperphagia [6]. Furthermore, Western diet causes a large increase in daily wheel running of HR mice (over many weeks), with little or no effect in C mice [68], demonstrating the importance of genetic history interacting with diet. More broadly, a better understanding of how eCBs in the gut respond to genetic and environmental factors may be essential to addressing issues of obesity, including non-communicable diseases (e.g., metabolic syndrome) to increased risk of infection from communicable diseases (e.g., COVID-19).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2021.113675.

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