ENDOCANNABINOID REGULATION OF HELMINTH INDUCED HYPOPHAGIA

By

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Abstract

More than 1.5 billion individuals are infected with soil-transmitted helminths globally. Helminths are parasitic worms which specifically travel into the gut where they alter nutrient absorption leading to malnutrition and anemia in hosts. While there is evidence indicating helminth infections lead to decreased weight as the host in consuming less, there is insufficient research on the mechanism through which this decreased appetite is induced. One system significantly involved in energy consumption is the endocannabinoid system, which consists of lipid signaling molecules that control feeding and metabolism. Hosts infected by helminths experience significant weight loss by day two of infection, which is recovered by day five post-infection. This study aims to identify brain endocannabinoid content in the hypothalamus, hippocampus, ventral and dorsal striatum of hosts infected with the soil-transmitted nematode Nippostrongylus brasiliensis at days 0, 2, and 8 post-infection. Endocannabinoid content will be quantified via ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). These data along with weight-loss may indicate how endocannabinoids influence feeding behavior in helminth infection. We hypothesize that if a decrease in endocannabinoid levels in the host's brain is responsible for reduced feeding, then we will see a down-regulation in endocannabinoid levels in the host's brain on day 2, and restoration to baseline by Day 8. Establishing that endocannabinoids play a key role in the feeding change expressed in hosts would allow for identification of a therapeutic treatment to minimize helminth infection associated weight loss and malnutrition.

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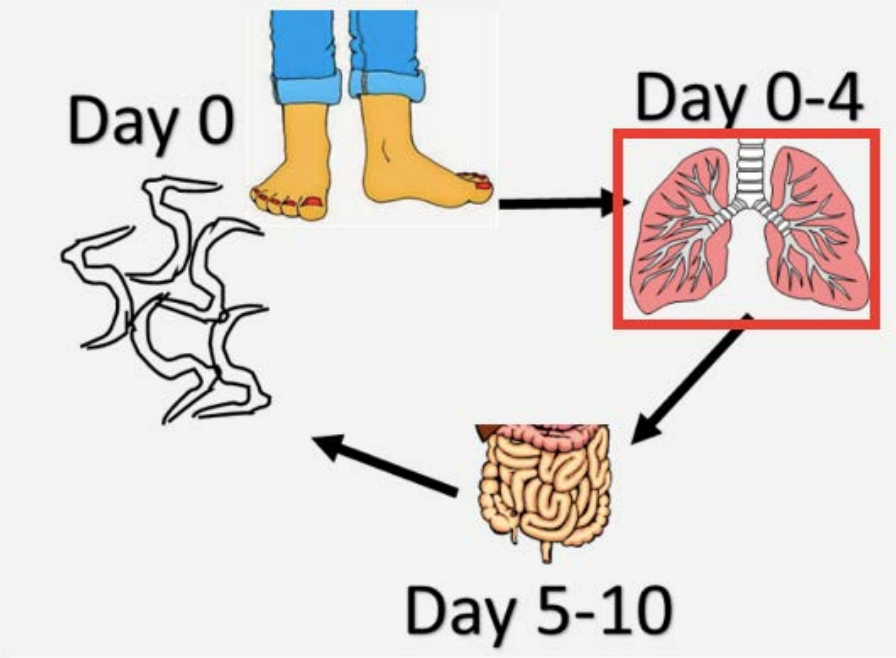
Introduction

More than 1.5 billion individuals are infected with soil-transmitted helminths globally (1). Helminths are parasitic worms which specifically travel into the gut where they alter nutrient absorption leading to malnutrition and anemia in hosts (1). These parasitic worms have a life cycle that has been sufficiently defined. In Figure 1, it is shown that on Day 0 of the infection, which occurs by crossing the skin barrier under the feet, the worm burrows through the skin toward the warmth of blood. Once inside the blood circulation, the helminths travel through the bloodstream to the lungs. Approximately 4 to 5 days post infection, the worm travels up the trachea and down the esophagus to the small intestine where the worm sexually matures and reproduces. The eggs of the worms are then released with the host's fecal matter, at which point the cycle repeats. Children infected by Helminths may experience growth retardation and physical impairments (1). These infections are particularly severe in impoverished countries that have inadequate healthcare and hygiene facilities (3).

Figure 1: Lifecycle of Helminths (Courtesy of Mark Wiley)



Recent findings indicate that mice hosts infected by helminths experience significant weight loss by day two of infection, however this is recovered by day five post-infection when the worms reach the small intestine (4). While there is evidence indicating helminth infections lead to decreased weight as the host is consuming less, however, there is insufficient research on the mechanism through which this decreased appetite is induced. One system significantly involved in energy consumption is the endocannabinoid system, which consists of lipid signaling molecules that control feeding and metabolism (5). It has also been recently shown that *Nippostrongylus brasiliensis* contains the necessary machinery to produce and degrade endocannabinoids and can serve as a model of human hookworm infection (6).

The endocannabinoid system has 2 receptor types including CB1 and CB2, to which endogenous agonists including 2-arachidonoyl-*sn*-glycerol (2-AG) and Anandamide (AEA) bind (7). 2-AG is known to modulate synaptic signaling via retrograde signaling. Once 2-AG is released, it acts on the presynaptic end leading to a reduction in neurotransmitter release (5). It is thought that AEA modulates local synaptic activity in a similar manner**.** Endocannabinoids including 2-AG and AEA can modulate activity by acting on multiple nuclei of the hypothalamus via CB1 receptors. (7). This suggests that endocannabinoids may be modulating the hypothalamus' integration of various signals about the body's energy levels. In a previous study, endocannabinoid administration directly inside the ventromedial nucleus of the hypothalamus lead to increased food intake through a CB1 receptor dependent mechanism (8). Additionally, pharmacological activation of the CB1 receptor has been shown to promote food consumption while reducing energy expenditure (9). It is well documented that central endocannabinoid activation of CB1 receptor promotes food consumption by inducing appetite, and increasing feeding behavior.

As weight loss is seen on day 2 post infection and by day 7 post infection this reduction in feeding is recovered, this study aims to identify brain endocannabinoid content in the hypothalamus and in the cerebellum of hosts infected with the soil-transmitted nematode *Nippostrongylus brasiliensis* at days 2 and 7 post-infection. Levels of 2-AG, AEA, and AEA's structurally similar endogenous analog OEA will be quantified via ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). These data along with weight-loss may indicate how endocannabinoids influence feeding behavior in helminth infection. We hypothesize that if a decrease in endocannabinoid levels in the host's brain is responsible for reduced feeding, then we will see a down-regulation in endocannabinoid levels in the hypothalamus of the host's brain on day 2, and restoration to baseline by Day 7.

Methods

Animals/Tissue Groups:

For this experiment, all mice groups where age matched between 6 and 8 weeks old, and consisted of C57Bl/6J Wild Type male mice. For this experiment we have 3 animal groups. One group consisted of 6 naïve mice which were not infected with *Nippostrongylus brasiliensis* and served as the baseline. A second group consisted of 6 infected mice from which brain samples were recovered 2 days post infection. Lastly our third group consisted of 6 infected mice from which brain samples were recovered 7 days post infection.

Table 1: Sample Numbers and Treatment Type

Sample Number	Infected/Naive	Days Post-Infection
1	Infected	7
2	Naive	7
3	Infected	2
4	Infected	2
5	Infected	7
6	Naïve	2
7	Infected	2
8	Naïve	2
9	Infected	7
10	Infected	2
11	Infected	2
12	Naïve	7
13	Naïve	7
14	Infected	7
15	Infected	7
16	Naive	2
17	Infected	2
18	Infected	2

Infection

The *Nippostrongylus brasiliensis* life cycle was maintained in female rats in the vivarium. *Nippostrongylus brasiliensis* eggs were collected from fecal matter after which they were cultured. Once the worms were approximately 10-20 days old, the mice were anesthetized using 2% isofluorene and infected with 600 infective *Nippostrongylus brasiliensis* larvae via subcutaneous injections.

Feeding Period/Weight Measurements

The mice were placed in their units 72 hours before the experiment to allow familiarization to the environment for all animal groups. All mice were given the same diet consisting of standard rodent chow and water. After this acclimation period, food and water intake was measured every minute in the feeding chambers and data analysis took place via TSE Phenomaster. Additionally, body weight data was collected daily and is shown in Figure 2 below.

Tissue Processing

At the time of harvest, specific brain regions were isolated and removed from anesthetized mice. The brain regions of interest, the cerebellum and the hypothalamus, were extracted from the mice brains and placed directed into liquid nitrogen for preservation. The brain samples taken from the mice were weighed to ensure similar tissue sample sizes were used. Special attention was given to the cutting of samples to ensure the same areas of the hypothalamus were being recovered, as much evidence has emerged in recent years suggesting that the endocannabinoid system can induce or inhibit feeding depending on the input type, and the cell or input type that the endocannabinoids are modulating (7).

These samples were then homogenized in 1 mL of MeOH with internal standards while being kept in an ice bath. The internal standards included d4-FAEs standard, d5-2AG 0.26 mM, and

19:2 DAG. Next, the lipids were extracted from these samples with 2 mL of chloroform and were washed with 1 mL of water. After all samples was centrifuged the samples at 4 ^{o}C, 3000 rpm for 15 minutes, the lower organic phases which held the lipid content were collected. Once the organic phases were collected, they were dried gently under 99.998% pure N_2 stream at 37^{o}C. The resulting dried pellet was then reconstituted in 2 mL of Chloroform. These samples were then vortexed again and separated further using silica gel column chromatography. The first eluate was collected, after which 2 mL of 9:1 Chloroform:Methanol was loaded into the columns. The second eluate was collected in the same vial as the first eluate. These collected eluates were then dried gently under 99.998% pure N_2 stream at 37^{o}C. Lastly, the dried pellet was resuspended in 200 µL of 1:1 methanol-chloroform and kept in screw top LC/MS vials with inserts.

*The protocols for animal care, use, and euthanasia were approved by the Institutional Animal Care and Use Committee at University of California, Riverside and were prepared according to the guidelines from National Institutes of Health.

Ultraperformance Liquid Chromatography-Tandem Mass Spectrometry

The lipid contents of the samples collected in this experiment were analyzed using Acquity I class Ultraperformance Liquid Chromatography with in-line connection to Xevo TQ-S micro-triple-quadrupole mass spectrometer with electrospray ionization sample delivery. The lipid contents were quantified with a stable isotope dilution method which detected proton or sodium adducts of molecular ions $[M + H/Na]^{+}$ in multiple-reaction monitoring (MRM) mode. Extracted ion chromatograms for MRM transitions were used to quantify the analytes of interest which

included 2-AG (*m/z* = 379.3 > 287.3), AEA (*m/z* = 348.3 > 62.0), OEA (*m/z* = 326.4 > 62.1), with their internal standards [$^2H_5$]2-AG (*m/z* = 384.3 > 93.4), [$^2H_4$]AEA (*m/z* = 352.4 > 66.1), and [$^2H_4$]OEA (*m/z* = 330.4 . 66.0). Along with the lipid samples, one blank sample was used for each brain region which was analyzed. The blank sample was processed and analyzed using the exact same methods and protocol as the rest of the samples, with the exception that no tissue was added to the blank. This blank served as a control, and no endocannabinoid contents were detected in our analysis.

Statistical Analysis

For statistical analyses, GraphPad Prism software was used**.** When three groups of data were being compared across one variable (Figures 4A, 4B, 5A, 5B**)**, one-way analysis of variance (ANOVA) was performed. For Figure 2 where two groups were being looked at across two variables, two-way ANOVA was performed. Lastly, when one variable was being looked at across two groups (Figure 3), a Student's t-test was performed. Statistical significance is indicated in Figures 2-5 as follows: * for $P \leq 0.05$; ** for $P \leq 0.01$; and *** for $P \leq 0.0001$.

Results

Section 1: Body Weight Data

In order to confirm the *Nippostrongylus brasiliensis* infection had occurred in accordance with the present literature, and to confirm the expected hypophagia manifested as decreased body weight on day 2 post-infection, body weight data was collected for the duration of up to 7 days post-infection in naïve and infected groups. On day 1 post-infection, we observed a significant reduction in energy consumption in the infected group compared to the naïve group. On day 2 post-infection, there was a significant decrease in body weight of the infected group compared to the naïve group.

Figure 2: Body Weight Data 0-7 Days Post-Infection

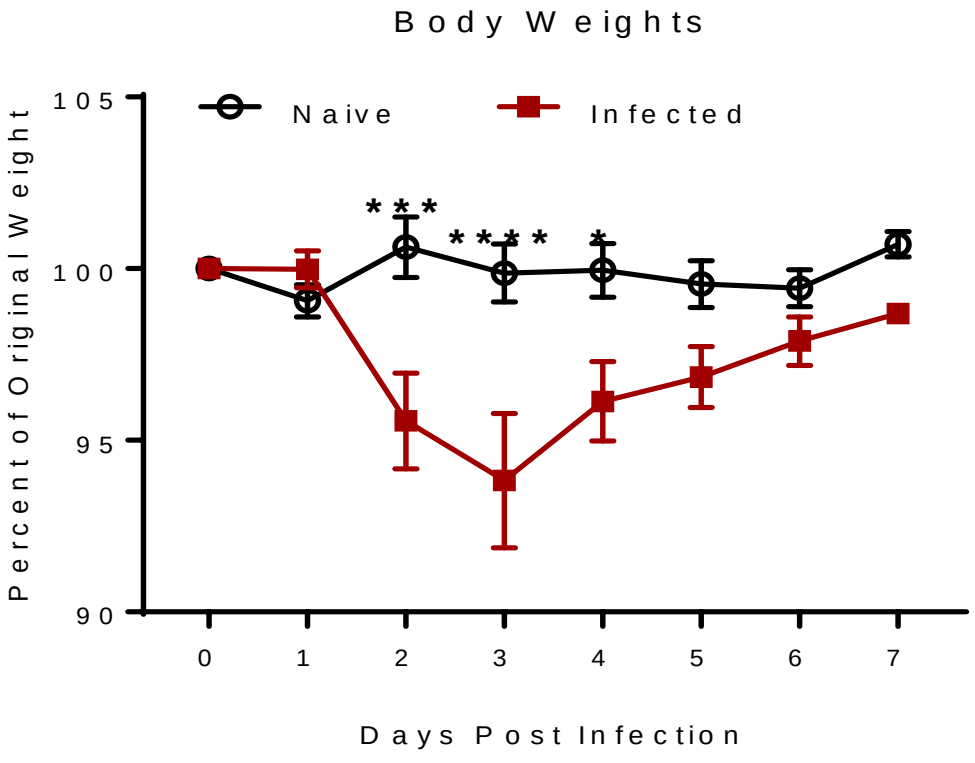


Figure 2 illustrates the body weight data which was collected daily from days 0 to 7 post-infection. The data for the naïve group is plotted in black, and the infected group is plotted in

red. The a significant decrease in body weight on day 2 post-infection as described in the literature. This decrease grows more significant on day 3 post-infection, but is eventually reduced.

Figure 3: Energy Consumption 1 Day Post Infection

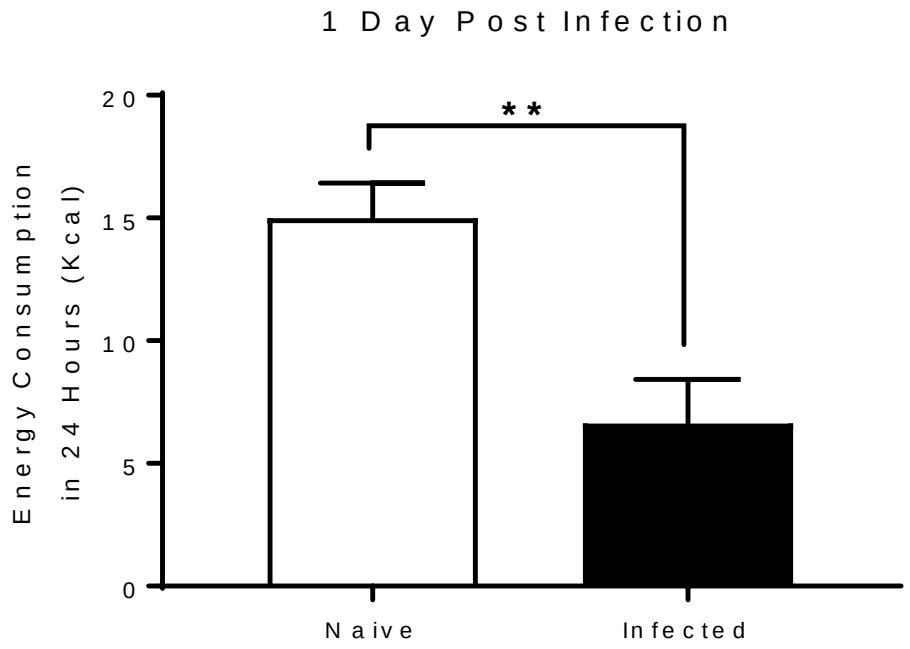
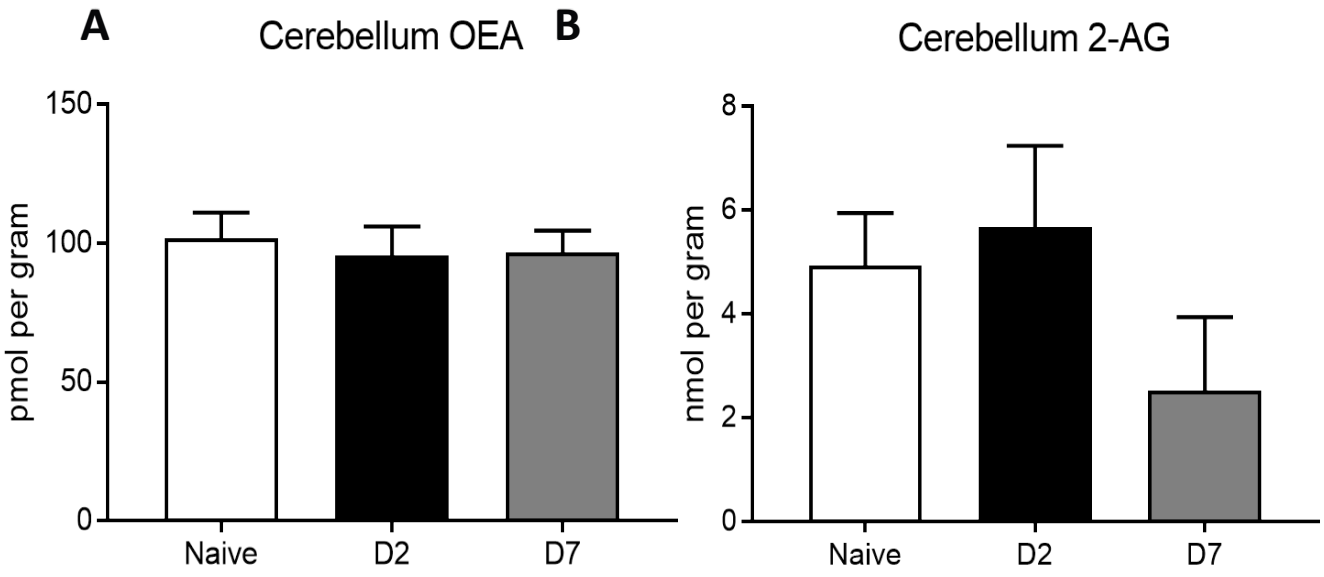


Figure 3 represents the total energy consumption in 24 hours on 1 day post infection. Daily energy intake was measured using the (system name). The naïve group's data is the white column which indicates 14.999 Kcal intake with a standard error of 1.439, and the infected group's energy consumption is represented by the black column, and represents 6.624 Kcal intake with a standard error of 1.800. The intake value for these two groups is significantly different, indicating marked reduction in energy consumption.

Endocannabinoid Content Quantification in Cerebellum and Hypothalamus

To assess if central endocannabinoids are modulating feeding behavior and promoting the onset of feeding reduction on day 1 post infection, and reduced body weight day 2 post infection, endocannabinoid content in the hypothalamus was measured. Alongside the hypothalamus, endocannabinoid content in the cerebellum was also quantified as an internal control as the cerebellum is not associated with energy regulation. In the cerebellum, no significant differences were observed between the naïve and infected groups in terms of OEA and 2AG levels. In the hypothalamus, the only significant difference was that OEA levels significantly increased on day 7 post-infection compared to day 2 post-infection. Very little AEA was observed in both brain regions.

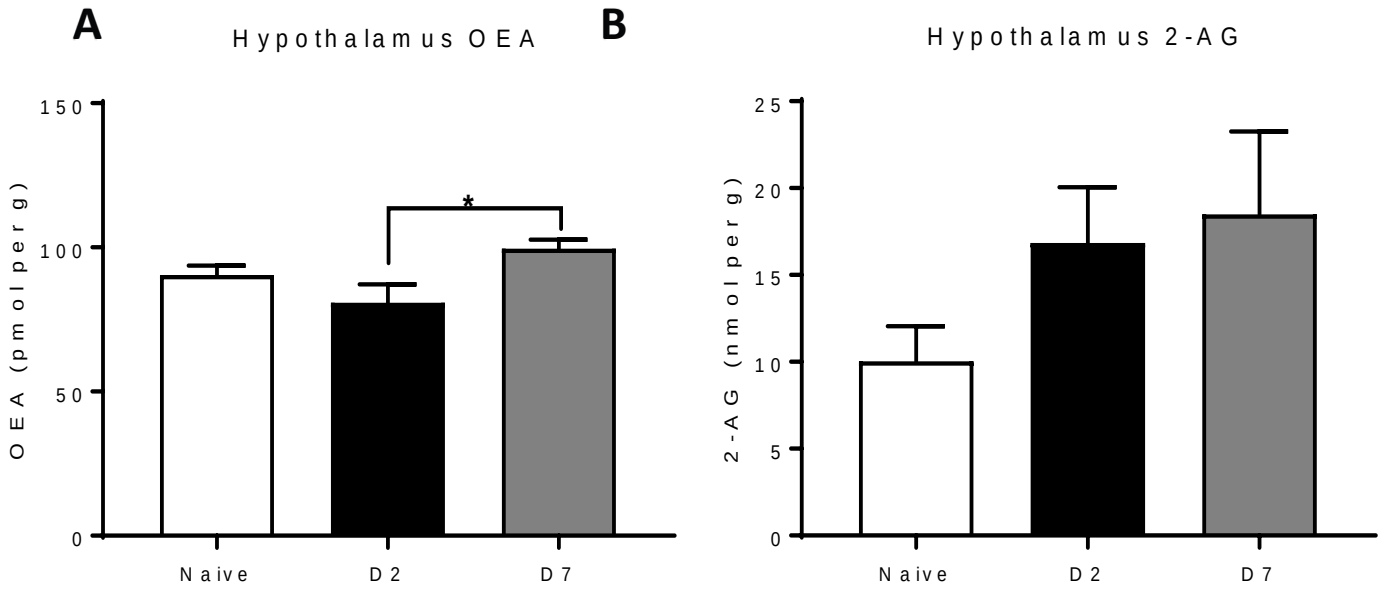
Figure 4: Cerebellum 2-AG and OEA Content



The endocannabinoid contents in the cerebellum of the naïve group, the day 2 post-infection group, and the day 7 post-infection group were quantified using ultra performance liquid chromatography coupled to tandem mass spectrometry. In Figure 4A, which shows the

cerebellum OEA levels, the naïve group had an average of 101.854 pmol with a standard error of 9.185. The infected samples recovered 2 days post-infection had an average of 95.504 pmol/gram with a standard error of 10.583. The infected samples recovered 7 days post-infection had an average of 96.727 pmol/gram with a standard error of 7.891. We see that OEA levels in the cerebellum remained similar across all three groups, without any significant differences. In Figure 4B, which shows cerebellum 2-AG levels, the naïve group had an average of 4.937 nmol/gram with a standard error of 1.005. The infected samples recovered 2 days post-infection had an average of 5.660 nmol/gram with a standard error of 1.575. The infected samples recovered 7 days post-infection had an average of 2.526 nmol/gram with a standard error of 1.415. Therefore, 2-AG levels in the cerebellum did not significantly differ between any of the three groups.

Figure 5: Hypothalamus OEA and 2-AG Content



The endocannabinoid contents in the hypothalamus of the naïve group, the day 2 post-infection group, and the day 7 post-infection group were quantified using ultra performance liquid

chromatography coupled to tandem mass spectrometry. In Figure 5A, which shows hypothalamic OEA levels, the naïve group had an average of 90.440 pmol/gram with a standard error of 3.260. The infected samples recovered 2 days post-infection had an average of 80.750 pmol/gram with a standard error of 6.445. The infected samples recovered 7 days post-infection had an average of 99.588 pmol/gram with a standard error of 3.139. While there was no significant difference between hypothalamic OEA levels of naïve and day 2 post infection samples, the hypothalamic OEA levels significantly increasing on Day 7 compared to Day 2 post infection. In Figure 5B, which shows the hypothalamic 2-AG levels, the naïve group had an average of 10.041 nmol/gram with a standard error of 2.005. The infected samples recovered 2 days post-infection had an average of 16.849 nmol/gram with a standard error of 3.210. The infected samples recovered 7 days post-infection had an average of 18.490 nmol/gram with a standard error of 4.762. 2-AG levels in the hypothalamus did not significantly differ between any of the three groups.

Discussion

Hosts infected by helminths experience significant weight loss by day two of infection, however this is recovered by day five post-infection according to recent findings (4). The literature also indicates that helminth infections leads to decreased weight as the host in consuming less. The body weight data for the naïve and infected groups showcased in Figure 2 is in accordance with the current literature as we observed significant weight loss 2 days post-infection in the infected group. After observing the body weight reduction, we further confirmed that the reduction in body weight is tied to a significant reduction in feeding behavior and energy intake. We can see that hypophagia occurred one day post-infection as there is a significant

reduction in energy consumption in the infected group compared to the naïve group. The body weight and energy consumption data being in accordance with the current literature indicate that our *Nippostrongylus brasiliensis* infection occurred successfully.

There is insufficient research on the mechanism through which this decreased appetite is induced and the endocannabinoid system seemed a possible candidate due to its complex regulation of feeding, energy consumption, and metabolism. We hypothesized that if a decrease in endocannabinoid levels in the host's brain is responsible for reduced feeding, then we will see a down-regulation in endocannabinoid levels in the host's brain on day 2 post-infection, and restoration to baseline by day 7 post-infection. We tested this hypothesis by quantifying endocannabinoid levels in the brain regions of interest, specifically the hypothalamus which integrates many signals and inputs to regulate feeding. Cerebellum endocannabinoid levels were also quantified to ensure that changes in central endocannabinoid levels were not broadly spread. From the data collected, we saw decent amounts of OEA and 2-AG, and very little amounts of AEA. OEA is structurally similar to AEA and is associated with satiety and anorexigenic effects while 2-AG is associated with food-seeking behavior (9). No significant differences were observed between the naïve and infected groups in terms of OEA and 2AG levels in the cerebellum or the hypothalamus. The only exception was hypothalamic OEA levels which significantly increased on day 7 post-infection compared to day 2 post-infection. None of this data supports the original hypothesis that a decrease in endocannabinoid levels in the host's brain is responsible for reduced feeding as we did not observe a down-regulation in endocannabinoid levels in the host's brain on day 2 post-infection. This indicates that the effects of hypophagia is not controlled by central endocannabinoids.

Controls were present at all steps of the experiment. The primary control was the naïve group which were age-matched, given the same diet, and held in the same environment. The only variable that was different was that the naïve group was not infected. Beyond the naïve group, an internal control was the cerebellum of the mice. While the hypothalamus was the region of interest as it is associated with regulating energy and feeding, the cerebellum is not associated with feeding regulation and thus served as a control. We quantified cerebellum endocannabinoid levels to ensure that changes in endocannabinoid levels were specific to the brain regions of interest and not broadly spread. To ensure that no contamination occurred during the homogenization and endocannabinoid quantification stages, a blank was used which was processed through the same equipment and only lacked a brain sample. Everything else was held constant. No lipid contents were detected in the blanks which indicates that cross-sample contamination did not occur.

While this study supports that there is a reduction in energy consumption on day 1 post infection, and there is a decrease in body weight observed day 2 post infection, this study suggests that central endocannabinoids do not influence the hypophagia observed in hosts infected with helminths. Moving forward, experiments can be conducted to identify other mechanisms which may be reducing energy consumption, or may be modulating this phenomenon. These may include studies looking at leptin and ghrelin signaling, or sympathetic regulation of brown adipose tissue thermogenesis, along with other circuits and signals tied to feeding behavior regulation and energy consumption and metabolism. The identification of the mechanism will allow for the development of therapeutic treatments. These treatments can lessen the magnitude of malnutrition and the associated growth retardation seen in extreme cases.

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