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Dietary Supplementation with Omega-3 Polyunsaturated Fatty Acids Reduces Opioid-Seeking Behaviors and Alters the Gut Microbiome

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Abstract: Opioids are highly addictive substances with a relapse rate of over 90%. While preclinical models of chronic opioid exposure exist for studying opioid dependence, none recapitulate the relapses observed in human opioid addiction. The mechanisms associated with opioid dependence, the accompanying withdrawal symptoms and the relapses that are often observed months or years after opioid dependence are poorly understood. Therefore, we developed a novel model of chronic opioid exposure whereby the level of administration is self-directed with periods of behavior acquisition, maintenance and then extinction alternating with reinstatement. This profile arguably mirrors that seen in humans, with initial opioid use followed by alternating periods of abstinence and relapse. Recent evidence suggests that dietary interventions that reduce inflammation, including omega-3 fatty acids such as docosahexaenoic acid (DHA), may reduce substance misuse liability. Using the self-directed intake model, we characterize the observed profile of opioid use and demonstrate that a diet enriched in polyunsaturated fat acids (PUFAs) ameliorates oxycodone-seeking behaviors in the absence of drug availability and reduces anxiety. Guided by the major role gut microbiota have on brain function, neuropathology, and anxiety, we profile the microbiome composition and the effects of chronic opioid exposure and DHA supplementation. We demonstrate that withdrawal of opioids led to a significant depletion in specific microbiota genera whereas DHA supplementation increased microbial richness, phylogenetic diversity, and evenness. Lastly, we examined the activation state of microglia in the striatum and found that DHA supplementation reduced the basal activation state of microglia. These preclinical data suggest that a diet enriched in PUFAs could be used as a treatment to alleviate anxiety induced opioid-seeking behavior and relapse in human opioid addiction.

Keywords: opioid; microbiome-brain axis; DHA; anxiety; polyunsaturated fatty acids; intravenous self-administration; mice

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1. Introduction

Several factors converged in the early 2000s to contribute to the escalating opioid epidemic. These included an over-prescription of potent and synthetic opioids, a belief that chronic pain was protective against the development of addictive behavior, an aggressive marketing strategy by the manufacturers, and the incorrect translation that long-term use of extended release opioids, safe in terminally-ill cancer patients, could be used in non-cancer patients without caution [1]. Alarming statistics from recent years document the increase in mortality from the use of fentanyl other synthetic opioids that are often prescribed for pain [2-5]. For many of these cases, the initial exposure to opioids began with oxycodone and other prescription analgesics and was then transferred to other more rapidly acting opioids [6]. We demonstrate in this paper that rapidly acting opioids are powerfully reinforcing and that dietary supplementation of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acids (DHA) may reduce the ability of these rapid-acting and potent compounds to maintain addictive-like behaviors via action on gut microbiome composition.

Whichever the opioid, an inherent property of these addictive substances is the high rate (~91%) of relapse [7] and the relative lack of effective treatment [8]. The rate of relapse following chronic opioid use has been linked to allostatic mechanisms that maintain persistent drug seeking such as a decline in cognitive control over habituated behaviors and tolerance to drug effects over time. The mechanisms that produce these long-lasting addictions are complex and can induce cravings that lead to relapse months or years after the physical opioid dependence is no longer a factor. According to the iRISA model of addiction proposed by Rita Goldstein and colleagues, the increased salience of drug-paired cues and impaired response inhibition in the absence of drug contribute to increased drug seeking over time and a persistence of seeking in the absence of drug [9-11]. These physical and emotional changes result in a persistent negative emotional state culminating in an inability to transition to an opioid-free state and to remain opioid-free [12]. Coupled with an increased vulnerability to stress and both physical and psychological stressors that trigger drug use in drug addicted humans and animals [13], relapse after prior opioid use is a common occurrence that must be addressed.

Several studies have focused on the bidirectional communication that takes place between the gastrointestinal tract and the central nervous system (CNS). Previous studies have revealed the major role gut microbiota have on brain function and consequently, neuropathology [14-18]. Anxiogenic affective-like behaviors are lower in mice that exhibit increases in specific genera including *Lactobacillus* and *Bifidobacterium* and these changes were linked to altered neural function [19, 20]. A similar relationship between *Bifidobacterium* and anxiety in humans has been established [21]. Still, the role of nutrition and supplementation in the microbiome-brain axis in psychiatry is not fully understood.

DHA is an essential long chain PUFA (DHA c22:6n-3) that is obtained from dietary sources and supplementation. The beneficial effects of supplementary DHA for many conditions and diseases have been the subject of ongoing research. A recurrent observation is that dietary omega-3 supplementation relieves anxiety and depression symptoms in mood disorders often co-diagnosed in substance abuse disorder patients (reviews; [22-24]. Accordingly, n-3 supplements enriched in DHA have been shown to reduce anxiogenic affective-like behaviors in preclinical models [22, 25, 26] and clinical trials [23, 27-31]. DHA is required as a structural component of plasma, microsomal and synaptic membranes in the brain [32, 33] and is essential for numerous brain functions including development [34], and diverse cellular functions [35-39]. There is also evidence that DHA supplementation has an effect on the gut microbiota, which can contribute to changes in brain function [40-42]. The overarching hypothesis for our study is that

DHA intake induces a beneficial shift in gut microbiome composition to normalize the genera that become overgrown with opioid-self administration.

We have previously shown that chronic, noncontingent, morphine induces diverse adaptations of the glutamatergic system in the striatum, a hub of the reward-centered mesolimbic circuitry [43]. Many of these cellular and behavioral adaptations were reduced or offset by a PUFA-enriched diet. The model of opioid exposure used in this previous study was an escalating dose of morphine, administered over 5 days by subcutaneous injection twice daily, followed an additional 3 days of morphine (at the highest dose) by a noncontingent method of delivery. In the present study, we used an opioid exposure model with greater predictive and face validity to mimic opioid use disorder. This model uses a contingent exposure whereby there is a choice as to how much opioid to self-administer through an indwelling intravenous catheter. The delivery of each drug infusion was contingent on pressing a designated active lever in an operant box. This allowed us to generate a profile of opioid use beginning with the acquisition of this behavior, followed by a period of maintenance, then periods of extinction alternating with reinstatement. This profile of opioid self-administration arguably recapitulates that seen in humans – with initial opioid use followed by alternating periods of abstinence and relapse. Using this model, we first outline this opioid use profile and then asked to what extent a PUFA enriched diet may alter drug-seeking behaviors. We also assessed to what extent the DHA supplementation had on affective-like behavior using a light dark test. We then examined how the chronic opioid self-administration and the DHA supplementation, separately and in combination, altered gut microbiome composition. We achieved this by examining the gut microbiome during multiple phases of the opioid exposure model. Lastly, we examined the state of the microglia in the striatum, a site we had previously studied [43], at the completion of the trial to determine if there was evidence of altered microglial activation due to the opioid exposure or DHA supplementation.

2. Materials and Methods

All the experiments were conducted in accordance with the AALAC Guide for the Care and Use of Laboratory Animals and approved by the UCLA IACUC committee (OARO #1999-179). Mice (C57Bl6/J Jax stock # 000664), 6–8 weeks of age at the start of the experiment, were randomly assigned to a control lab chow diet containing 0.5% DHA (Control) alone or supplemented with 2.5% DHA, 1.1% EPA and 0.75% other omega-3 PUFAs, (Nordic Naturals, Watsonville, CA) for 8 weeks. As we have previously shown a relative lack of effect of this DHA supplementation protocol on DHA enrichment in specific brain regions in female C57Bl6/J mice [43], only male C57Bl6/J mice were used for these experiments. Where possible, experimenters were blind to treatment and diet for both behavioral testing and data analyses.

Intravenous self-administration (IVSA) administration. Mice were divided into 4 groups: control diet + saline (n=14), control diet + opioid (n=14), DHA diet + saline (n=5), and DHA diet + opioid (n=10). An intravenous catheter (0.2 mm i.d., 0.4 mm o.d., Cathcams, Oxford, UK) was inserted into the right jugular vein of mice under sterile conditions as others and we have previously described [44-46]. Post-operative care included Carprofen gel food (MediGel CPF, Portland, ME, USA). Catheters were flushed daily with 0.02 ml Heparin/saline (30 USP/ml). The overarching timeline of experiments is described in Figure 1. Mice were monitored twice daily for the first 48h after surgery and exclusion criteria of more than a 15% weight lost or a moribund state for over 24h were applied. Catheter patency was tested using an infusion of propofol (20 µl of 1% propofol w/v in saline) every five days. After 3 days of postoperative recovery, mice were trained to lever press in the self-administration operant boxes (Med-Associates Georgia, VT, USA) by using a droplet of 20% sweetened condensed milk placed above both the active (AL) and inactive (IAL) levers (3x per session) during the first two 120 min sessions. Mice underwent daily 2h self-administration sessions where the active lever was paired with remifentanil and inactive lever was paired with saline infusion

using a random lever-infusion assignment (Figure 1). An AL press resulted in an intravenous drug infusion (0.67µl/g body weight) and the presentation of a 10s tone and visual cue (light), together these comprise a "reinforcer" (RNFS). A 10-s 'timeout' period followed each RNFS during which time no further RNFS could be earned. The mice first underwent a minimum of 3 days of acquisition training using remifentanil (0.05 mg/kg/infusion) as the delivered opioid at a fixed ratio of one drug infusion for each lever press (FR1) to a maximum of 50 RNFS or 2h, whichever came first (the acquisition phase). Once the criterion of 50 RNFS or 20% stability was obtained for 2 consecutive days, the mice were transitioned to oxycodone (0.25 mg/kg/infusion) under the same short-access 2h FR1 schedule for 10 days (the maintenance phase). This was followed by 5 days of extinction (1E) in which no further drug was delivered in response to AL-pressing but the same environment and reward-associated cue conditions were presented but with no limit on the number of RNFS that could be earned with the session ending after 2h. This was followed by a 2-day period of reinstatement in which oxycodone was administered intravenously at the same dose and cues as used during maintenance. Thereafter the mice underwent a second extinction for an additional 5 days under the same conditions as during the first extinction with no limit on the number of RNFS that could be earned and the session ending after 2h (2E). Due to the loss of catheter patency, the number of mice in these later stages declined from those at the beginning of the experiment. *Statistical Analyses.* The inter-and intra-session data were analyzed as a function of group and time using a linear mixed model in R (R Core Team, 2015) using the package "lmerTest" [47]. The linear models were generated for each experimental group of interest. The models were used to assess the effect of time, diet group, or an interaction of these factors on AL presses and RNFS earned. Whenever a significant effect was observed, a new reduced model was generated by removing the significant factor and compared with the original model using an ANOVA in order to assess the impact of the respective factor on the goodness-of-fit for the model. The resulting model is a regression equation where the intercept is allowed to vary for each subject: $Y_{Characteristic} = \beta_0 + \beta_{Group}\ X_{Group} * \beta_{Time}\ X_{Time} + U_{Subject}$ where $Y_{Characteristic}$ is the characteristic being modeled (e.g., active lever presses, reinforcers earned), each predictor variable is represented by its subscripted X, and $U_{Subject}$ represents the random effect of each individual subject. The coefficients (β) are estimated and assessed for significance and the contribution to the goodness of fit of the model was assessed.

Light dark assay. The apparatus consisted of light and dark compartments of a square box (28cm^2 square 18cm height) separated by a guillotine door in a quiet room illuminated at 50-55 lux. The light compartment was illuminated at 1000 lux by a halogen lamp, measured at the guillotine door. Mice (control diet; n=7, DHA diet; n=7) were placed in the dark compartment with the door closed for 2 minutes after which the guillotine door was raised to allow free movement between the light and dark compartments for an additional 2 minutes. Video tracking was recorded by a A1300-60gm Basler ace camera (106580-08, Basler, Germany), saved by the downloadable VLC video software (VideoLAN) and analyzed for the time for a full body exit from the dark compartment by experimenters blind to diet and opioid treatments. *Statistical Analysis.* Data were analyzed by Linear Mixed Models as previously described [48]. This test was conducted 3 days before insertion of the intravenous catheter and just before the operant session on the second day of the second extinction period (25 days after the initial test).

Microbiome characterization: 16S ribosomal RNA sequencing. Stool collection was done at the following time points: baseline (control diet; n=5, DHA diet; n=3), 1st day of oxycodone IVSA (control diet; n=11, DHA diet; n=11), 10th day oxycodone IVSA (control diet; n=15, DHA diet; n=10), 5th day of first extinction (control diet; n=15, DHA diet; n=10), and 1st day of second extinction (control diet; n=7, DHA diet; n=7). Stool was collected a minimum of 30 minutes before any behavioral tests, including IVSA. Stool was collected fresh, and did not interact with any non-sterile surfaces. Mice were placed on top of cage (to provide them with something to hold onto), held gently at the base of their tails and massaged until stool started to be naturally expelled. Once stool was partly exposed, sterile forceps was used to remove the stool and place it into a

sterile tube. Within 2 minutes of extraction, they were placed in an -80°C freezer and stored for 2-3 months prior to 16S rRNA sequencing. DNA was extracted from frozen fecal pellets using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) with bead beating following the manufacturer's instructions. The V4 region of 16S ribosomal RNA genes was amplified and underwent 2x150 sequencing on an Illumina MiSeq as previously described [49]. The base pair reads were processed using QIIME v1.9.1 with default parameters [50]. Sequence depth ranged from 47,843 to 128,236 sequences per sample. Operational taxonomic units (OTUs) were picked against the May 2013 version of the Greengenes database, pre-filtered at 97% identity. OTUs were removed if they were present in less than 10% of samples. Alpha diversity (i.e. diversity within a sample) and beta diversity (differences in composition across samples) were calculated in QIIME using OTU-level data rarefied to 47,843 sequences. *Statistical Analyses.* The significance of differences in alpha diversity metrics - Faith's phylogenetic diversity (Faith's PD), Chao1, and Shannon index - was calculated by analysis of variance. Beta diversity was calculated using square root Jensen-Shannon divergence and visualized by principal coordinates analysis. Adonis, a permutational analysis of variance, was performed using 100,000 permutations to test for differences in square root Jensen-Shannon divergence distances across diet and groups[51]. Association of microbial genera with diet and group (oxycodone and extinction phase) were evaluated using DESeq2 in R, which employs an empirical Bayesian approach to shrink dispersion and fit non-rarified count data to a negative binomial model [52]. After DESeq2 analysis, it was seen that a few mice in the colony were affected by an overgrowth of segmented filamentous bacteria (i.e. *Candidatus Arthromitus*). Because this represented a contaminant genus unrelated to the experimental interventions, it was excluded from analysis. P-values for differential abundance were converted to q-values to correct for multiple hypothesis testing (< 0.05 for significance) [53].

IBA1 and CD68 labeling. Brains from the four testing groups including the control diet + saline (n=2), control diet + oxycodone (n=2), DHA diet + saline (n=2), and DHA diet + oxycodone (n=5) were collected 48h after the last operant session. They were then placed in 4% paraformaldehyde overnight at 4°C followed by 24-48h in 30% sucrose until equilibrated, then frozen in a dry ice/ isopropanol bath and stored at -80°C. Fifty µm sections were later cut and collected into phosphate buffered saline (PBS), washed in PBS with 0.1% Triton X-100 (Sigma, MO, PBS-T) 3 x, 10' each, and blocked for 2h at RT in 5% NGS + 3% BSA in PBS-T. They were then incubated in the following antibodies diluted in the wash buffer; anti-rabbit IBA-1 (1;2,000, Wako, Richmond VA, cat. # 019-19741) and anti-mouse CD68 (1:1000, BioRad Hercule, CA, USA, cat. # MCA 1957) overnight at 4°C. After another 3 washes for 10' each, sections were incubated in the following secondary antibodies; goat anti-rabbit 488 (1:1000) and goat anti-Rat 647 (1:1000, both from Thermofisher, Waltham, MA) diluted in PBS-T, for 2h at RT. After a final 2 washes in PBS-T and one in PBS for 10' at RT, the sections were mounted on Permamount slides and coverslipped with a DAPI Antifade mounting medium (VectorLabs, Burlingame, CA). A Leica DM5500 B upright microscope with a Leica DFC9000 GT sCMOS camera and LAS X software (Lieica, Germany) and a 20x objective was used to obtain tiled images of each section. Sections (10x10") from dorsomedial and dorsolateral regions of the striatum were exported to ImageJ [54]. *Statistical Analyses.* The shape and intensity of CD68 labeled cells was quantified by a user blind to the group identity with 5-30 cells quantified in each image and 5-10 images quantified for each of the dorsolateral and dorsomedial striatal sections of each mouse, and as there was no effect of region, they were combined into a single dataset for analysis by one-way ANOVA (Prizm v8).

3. Results

3.1 The experimental timeline.

A schematic of the timeline used is shown in Figure 1 and described as follows. The basal light dark test and fecal collection occurred prior to the IVSA surgery which was then followed by the IVSA self-administration protocol consisting of 5 sequential phases; acquisition, maintenance, extinction, reinstatement, and a second extinction. Fecal collection was performed between each of these phases with the final light dark test taking place on day 24 during the second extinction. The experiment terminated with the collection of brain tissue on day 27 **(Figure 1)**.

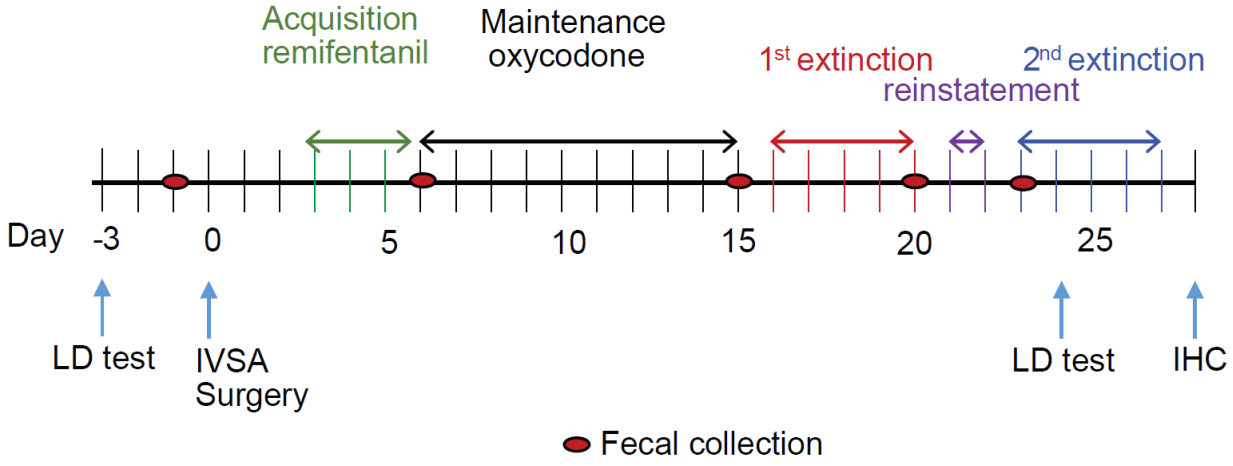

Figure 1. The Experimental Timeline. This schematic shows the type and order of interventions in the 27-day protocol used for mice that had received a DHA-enriched or control diet for 8 weeks prior to the start of the protocol. Following the implant of the jugular catheter (IVSA surgery), mice underwent daily sessions where, after acquisition of remifentanil self-administration, oxycodone or saline infusion was self-administered for 10 days (the maintenance phase) followed by 5 days of extinction (extinction 1, 1E) in which no further drug was delivered but the same environment and reward-associated cues were presented. This was followed by a 2-day period of reinstatement (reinstatement) in which oxycodone was administered at the same dose and cues as used during maintenance. Thereafter the mice underwent a second extinction (extinction 2, 2E) over the following 5 days under the same conditions as during 1E. Fecal boli were collected before the surgery and at each change in this protocol as indicated. The light dark (LD) test was conducted before the surgery and on the 2nd day of 2E. Brain tissue was collected at the end the protocol for microglial analysis by immunohistochemistry (IHC).

3.2 A DHA-enriched diet reduces oxycodone-seeking behaviors.

To both characterize the opioid self-administration profile and to assess the effect of a DHA-enriched diet on this profile, mice maintained on a standard laboratory chow diet were compared to mice given an increased level of DHA supplemented into the food. Both groups of mice were trained to self-administer opioids in order to analyze drug specific behaviors, the first when remifentanil was self-administered **(Figure S1A-S1B)** and the second when oxycodone was self-administered (**Figure 2A-2F)**.

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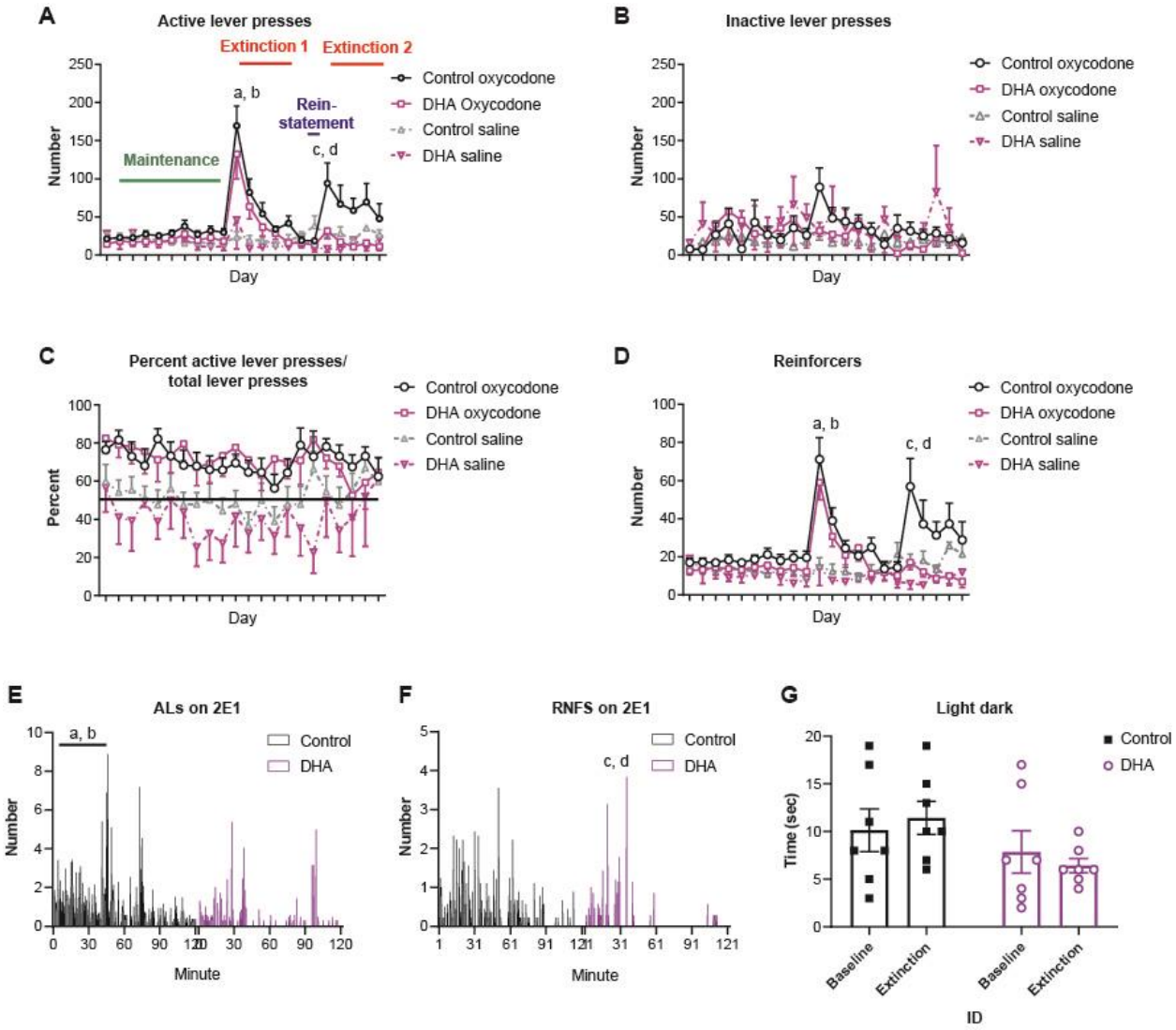

Figure 2. A DHA enriched diet reduces oxycodone seeking behaviors. **(A) Active Lever Presses**. Mice on both the control diet (black) and the DHA diet (purple) showed an increase in AL pressing on the first day of 1E (a; $p<0.0001$) and a decline in lever pressing thereafter (b; $p<0.0001$). AL pressing during reinstatement did not differ from the last day of 1E training or maintenance. However, during 2E there was an increase in lever pressing above that of reinstatement (R1 or R2 vs 2E1; c; $p<0.01$) for the control mice but not for the DHA mice. Furthermore, active lever pressing by the DHA mice was lower than that of the control mice for all days of 2E (d; $p<0.05$). **(B) Inactive lever presses.** There was no effect of diet or day on this parameter **(C) Percent active lever presses.** Although both saline groups showed ≤ 50% accuracy in AL presses as a percent of total lever presses this was not different from both opioid groups with≥ 50% AL presses. **(D) Reinforcers**. Control diet mice earned more RNFS on 1E1 (a; $p<0.001$) and this declined during the remaining days of 1E (b; $p<0.0001$). The number of RNFS earned by the controls again increased above that of reinstatement during 2E (R1 or R2 vs 2E1; c; $p<0.01$). DHA mice had a similar initial profile as those on the control diet with an increase in RNFS earned on 2E1(a; $p<0.0001$) and a decline in RNFS earned for each day of 1E thereafter (b; $p<0.0001$). However, the 2E1-5 sessions did not induce an increase in RNFS earned from that seen during reinstatement. **(E) Active Lever Presses during 2E1**. During the first hour of this session the control mice pressed the AL more than DHA mice (a; $p<0.05$) and this rate declined during the second hour (b; $p<0.05$).**(F) Reinforcers during 2E1.**During the second but not the first hour the number of RNFS earned

declined more rapidly in the DHA than control mice (DHA vs control diet; c; $p<0.01$, and an effect of time in DHA but not control mice d; $p<0.01$). **(G) Light Dark Test.** Although the DHA intervention did not alter the basal levels of anxiety, this diet did reduce anxiety, reflected as a quicker entry into the light compartment from the dark compartment by the DHA group on 2E2 (a; $p<0.05$ vs extinction of the control mice).

Mice underwent at least three days of acquisition training using remifentanil as the delivered opioid on an FR1 schedule. There was no effect of diet in the initial acquisition of opioid self-administration; using remifentanil as the reinforcer, neither active lever presses ($F_{(2,88)}=1.185$, $p=0.3$) nor reinforcers ($F_{(2,88)}=0.837$, $p=0.44$) were significantly different between diet groups (**Figure S1A-S1B)**.

The number of AL presses across this profile showed an effect of day ($p<0.001$, $\chi^2 = 12.147$) and diet ($p<0.05$, $\chi^2 = 4.646$), as well as, a significant day-by-diet interaction ($p<0.01$, $\chi^2 = 7.526$). For mice on the control diet, post-hoc analysis revealed an increase in AL pressing on 1E1 ($p<0.0001$, $\chi^2 = 21.367$) and a decline in lever pressing thereafter during sessions 2E2-5($p<0.0001$, $\chi^2 = 31.481$). AL pressing during reinstatement did not differ from the last day of the first extinction period (1E), but again increased on the first day of 2E following the 2-day reinstatement of oxycodone (R1 or R2 vs 2E1; $p<0.01$, $\chi^2 = 7.164$). For mice on the DHA diet, post-hoc analysis showed an initial similarity as those on the control diet with an increase in lever pressing on 1E1 ($p<0.01$, $\chi^2 = 10.583$) and a decline in lever pressing for each day of 1E thereafter ($p<0.0001$, $\chi^2 = 20.022$). However, the second extinction period following the 2 reinstatement sessions did not induce an increase in ALs. Furthermore, AL pressing across sessions 2E1-5 by the DHA mice was lower than that of the control mice ($p<0.05$, $\chi^2 = 5.136$) (**Figure 2A**). These results are consistent with the DHA-enriched diet reducing oxycodone seeking behaviors during the second but not first extinction period.

In assessing goal-directed lever pressing behavior, the number of inactive (no drug infusion) lever presses showed significant day-by-diet interaction ($p<0.05$, $\chi^2 = 4.775$); however, there was no independent effect of diet, ($p=0.70$, $\chi^2 = 0.143$) or day ($p=0.58$, $\chi^2 = 0.305$) on this parameter (**Figure 2B**). Goal-directed lever pressing behavior can also be evaluated by the percent of active lever presses out of the total lever presses (AL/(AL + IAL)). This showed an effect of day ($p<0.0001$, $\chi^2 = 19.231$), but not diet ($p=0.67$, $\chi^2 = 0.178$) nor any day-by-diet interaction ($p=0.69$, $\chi^2 = 0.156$) (**Figure 2C**).

A press on the AL followed by the delivery of oxycodone or saline, a tone and 10s visual light cue, and a 10s time-out period together comprised the reinforcer (RNFS). When measuring the number of RNFS earned, we found that this parameter mirrored the AL presses in that there was a day ($p<0.01$, $\chi^2 = 10.361$) and diet effect ($p<0.05$, $\chi^2 = 4.694$), as well as a day-by-diet interaction ($p<0.01$, $\chi^2 = 9.726$). Further post-hoc analysis showed that, for mice on the control diet, the number of RNFS earned increased on 1E1 ($p<0.0001$, $\chi^2 = 16.547$) and declined thereafter ($p<0.0001$, $\chi^2 = 25.288$). The number of RNFS obtained during reinstatement did not differ from the last day of E1 and were lower than the last day of maintenance ($p<0.01$, $\chi^2 = 10.538$). However, there was an increase in RNFS earned on the first day of the second extinction period (2E1) above that of reinstatement (R1 vs 2E1; $p<0.01$, $\chi^2 = 7.790$). For mice on the DHA diet, post-hoc analysis revealed an initial similarity with those on the control diet showing an increase in RNFS earned on 1E1 ($p<0.0001$, $\chi^2 = 17.498$) and a decline in RNFS for each day of 1E thereafter ($p<0.0001$, $\chi^2 = 20.478$). However, the 2E1-5 sessions did not induce an increase in RNFS earned when compared to the level observed during reinstatement. Furthermore, RNFS earned by the DHA mice across the five days of this 2E period were lower than that of the control mice ($p<0.01$, $\chi^2 = 6.842$) (**Figure 2D**).

Given our observation that the first day of 2E showed a clear protective effect for the DHA diet on opioid seeking behaviors, we then focused on the within-session behavior. Specifically, we analyzed the rate of AL pressing or RNFS earned within the 2 h access window on the first day of 2E. Using mixed models linear analysis of these datasets we found that the rate of AL pressing showed an effect of diet ($p<0.05$, $\chi^2 = 3.889$), time ($p<0.0001$, $\chi^2 = 30.788$), and an interaction of diet and time ($p<0.01$, $\chi^2 = 6.800$) with the control group pressing more frequently than the DHA group (**Figure 2E-2F**).Further in-depth analysis of the hourly data showed an effect of diet ($p<0.05$, $\chi^2 = 4.018$), but not time, on the frequency of AL pressing with the control group pressing more during the first hour. During the second hour, the frequency of AL pressing declined resulting in an effect of time ($p<0.05$, $\chi^2 = 4.155$), and a diet-by-time interaction ($p<0.05$, $\chi^2 = 4.884$), but no effect of diet alone (**Figure 2E**). In contrast, the rate of RNFS earned did not show any clear effect of diet, time, nor an interaction between the control and DHA diet for the entire 2h session. Post-hoc analysis of each hour revealed no significant difference in RNFS earned during the first hour. However, there was an effect of diet ($p<0.01$, $\chi^2 = 7.254$), time ($p<0.01$, $\chi^2 = 8.041$), and a diet-by-time interaction ($p<0.001$, $\chi^2 = 10.993$) for the second hour. This was due to the rate of RNFS earned by the DHA mice declining more rapidly than the control group during this hour (**Figure 2F**).

3.3 The DHA-enriched diet reduced anxiety-like behavior during extinction.

We have previously shown that a DHA-enriched diet reduces the anxiety state of mice following chronic morphine [43]. Here we used the well-established light-dark test to repeatedly assess the state of anxiety [48] [55] (**Figure 2G**). Using linear regression analysis we found that, although the DHA diet did not alter the basal levels of anxiety ($p = 0.4845$, $F_{[1,12]} = -0.5203$), that the DHA-enriched diet reduced anxiety-like behavior as assessed by decreased latency into the anxiogenic light compartment ($p<0.05$, $F[1,12] = 7.027$).

3.4 Microbiome profiles change with a DHA-enriched diet and with opioid extinction.

We characterized the effect of DHA on gut microbiome composition in the context of opioid exposure by performing 16S rRNA sequencing of fecal samples collected from the two dietary groups at baseline, during oxycodone maintenance (days 1 and 10), 1E, and 2E. There was a significant difference in overall microbial composition between mice on the control diet and mice on the DHA-enriched diet (p-value <0.05) after adjusting for study phase (**Figure 3A**). There was also a significant difference in the microbiome during the oxycodone maintenance phase as compared to both extinction group periods (1E & 2E) while controlling for diet (p-value <0.05). There was no statistical difference between microbiome profiles at day 1 (D1-OXY) of oxycodone maintenance and day 10 (D10-OXY). There was also no statistical difference in overall microbial composition between 1E and 2E. DHA supplementation lead to a significant increase in species richness measured by the number of types of organisms (Chao1, p-value =0.01), phylogenic diversity measured by the evolutionary distance between organisms (Faith's PD, p-value =0.003), and species evenness measured by the abundance of organisms across species (Shannon Index, p-value=0.01) (**Figure 3B**).

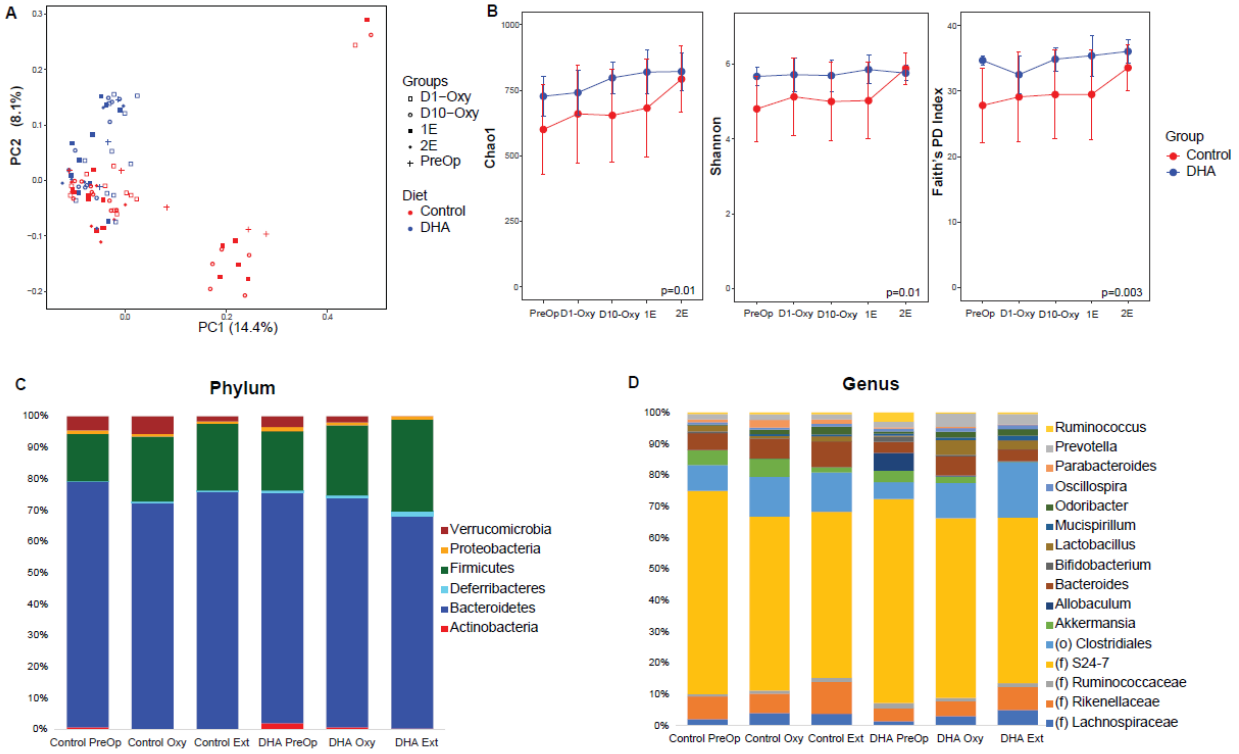

Figure 3. DHA supplementation and opioid extinction alter gut microbiome profiles. (A) Principal coordinate analysis plot separated by diet and study phase (D1-OXY: Day 1 of oxycodone; D10-OXY: Day 10 of oxycodone; 1E: First extinction phase, 2E: Second extinction phase). Each symbol represents one fecal sample, with color denoting dietary group and shape denoting phase. The axis labels indicate the percentage of variation represented by each coordinate. **(B)** Microbial composition and diversity. Species richness (Chao1) (left), species evenness (Shannon Index) (middle), and phylogenetic diversity (right) by diet (control diet in red and DHA enriched diet in blue). **(C)** Phylum and **(D)** genus summary by groups. Bar plots show the average relative abundance of microbes at the phylum and genus levels. D1-OXY and D10-OXY were combined, as were 1E and 2E.

Differential abundance testing was performed at the genus level to identify microbes that were associated with DHA supplementation during the oxycodone maintenance and opioid extinction phases using DESeq2 models at a 5% false discovery rate threshold (q-value<0.05). DHA supplementation lead to a significant decrease during the oxycodone maintenance phase in *Akkermansia* (4-fold) and *Parabacteroides* (5.5-fold) and a significant increase in multiple genera including *Lactobacillus* (8-fold), *Allobaculum* (7-fold) and *Bifidobacterium* (7-fold) (**Figure 4A**). During the opioid extinction phase, DHA supplementation was similarly associated with depletion of *Parabacteroides* (3.4-fold) and enrichment of *Bifidobacterium* (8.5-fold), but *Lactobacillus* was not significantly different and additional differences were observed including increased *Desulfovibrio* (6-fold) (**Figure 4B**). Similar analysis was then performed to identify genera associated with the opioid extinction phase compared to the oxycodone maintenance phase in one or both diets. Opioid extinction led to a significant decrease in *Akkermansia* (64-fold) and *Bifidobacterium* (8-fold) independent of diet. Opioid extinction also led to a significant decrease in *Parabacteroides* (5-fold) but only within the control diet group (**Figure 4C-4D**). *Lactobacillus* did not significantly change with opioid extinction.

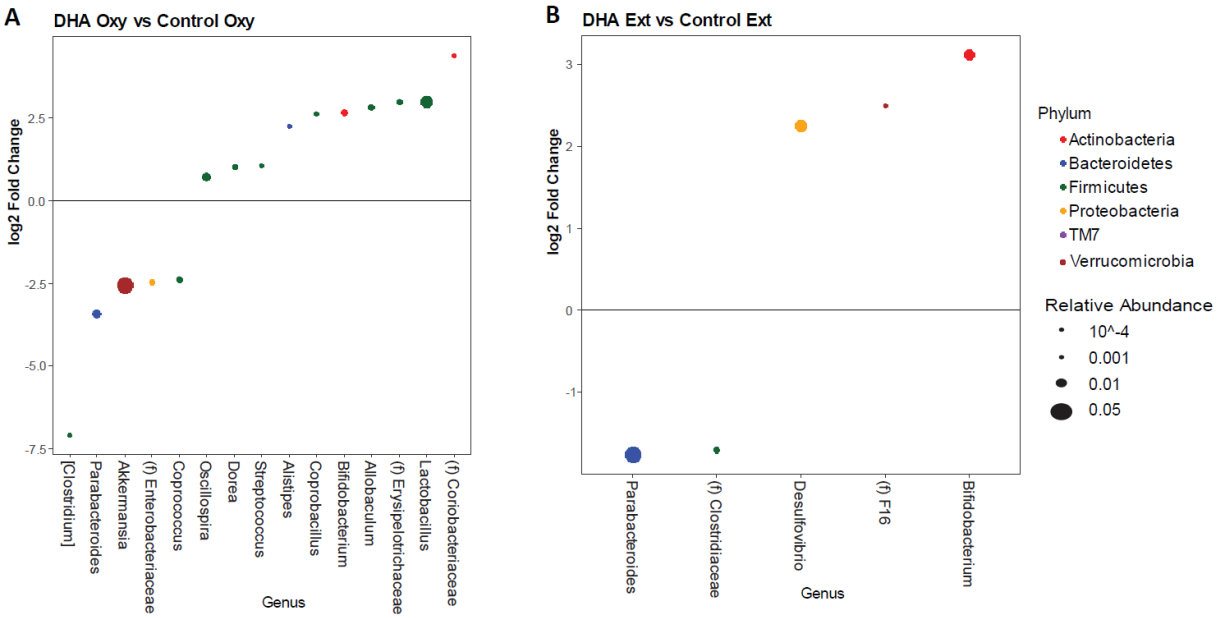

Figure 4: Differential abundance of microbes differ by DHA supplementation and opioid extinction. (A) Effects of DHA on genera abundance during oxycodone maintenance. DESeq2 models were used to identify differentially abundant genera ($q<0.05$) in the DHA supplementation group, combining data from D1-OXY and D10-OXY and adjusting for time point. Differences are expressed as log2 fold change, with dot size proportional to mean genera abundance across samples and dot color indicative of phylum. **(B)** Effects of DHA on genera abundance during opioid extinction. DESeq2 analysis of combined 1E and 2E data, adjusted for phase. **(C)** Effects of opioid extinction on genera abundance while on DHA supplementation. **(D)** Effects of opioid extinction on genera abundance while on control diet.

3.5 The activation state of microglia is affected by the DHA-enriched diet but not opioid IVSA.

To assess the activation state of microglia we labeled these cells with 2 antibodies, one against CD68, a lysosomal protein expressed in high levels in activated microglia [56] and one against IBA1, a calcium binding protein found in microglia and macrophages [57]. This enabled us to count the number of CD68-labeled microglia in the dorsomedial and dorsolateral striatum and then assess the shape and density of IBA1 labeling in these CD68-positive cells. A representative slice image is included in **Figure 5A**.

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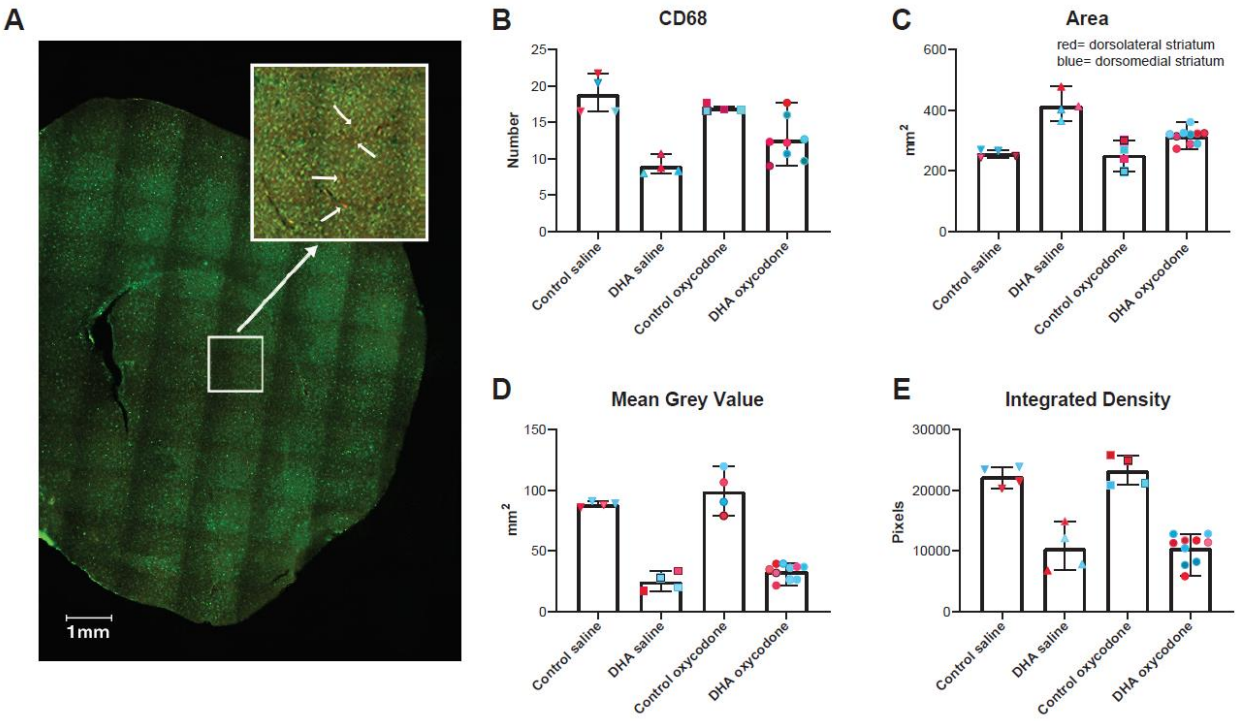

401 **Figure 5. The activation state of microglia is affected by the DHA-enriched diet but not by** oxycodone
402 **self-administration.** To assess the activation state of microglia, the number of CD68-labeled cells in the
403 dorsomedial and dorsolateral striatum were counted and the shape and density of IBA1 labeling in these
404 CD68+ cells was assessed. There was no effect of region on any of these parameters so the dorsomedial and
405 dorsolateral data were combined. **(A)** A representative example of a coronal section with CD68 labeled in
406 red and IBA1 in green. An example of the dorsolateral region is enlarged with the arrows showing cells that
407 are labeled with both the CD68 and IBA1 antibodies. **(B) CD68**.The DHA dietary regimen reduced the
408 number of CD68+ cells compared to the control group ($p<0.001$) but opioid IVSA did not induce any further
409 change. **(C) Cell area.** The area of the cells of the controls, whether saline or opioid-treated, was lower than
410 that of the DHA control (***, $p<0.001$) or opioid-treated (*, $p<0.05$) groups. **(D) Mean grey value or average**
411 **pixel density.** This parameter showed an effect of treatment with the DHA groups, whether saline or opioid-
412 treated, showing less pixel density than the control groups (***, $p<0.001$ in both groups). **(E) Integrated**
413 **density or the sum of the pixels in the selected area.** This parameter was similarly altered by treatment
414 with an effect of DHA (***, $p<0.001$) but no effect of opioid exposure.

415 The DHA group showed a basal reduction in the number of CD68+ cells as compared to the control
416 diet group ($F_{(3, 16)} = 14.66$, $p<0.001$; one-way ANOVA), but the opioid self-administration did not induce
417 any further change (**Figure 5B**). We next analyzed cell morphology by assessing cell area for the CD68+
418 cells. The cell area of the control diet group, whether saline or opioid-treated, was lower than that of the
419 DHA control ($p<0.001$) or opioid self-administration ($p<0.05$) groups. There was no effect of opioid self-
420 administration in the control diet group, but opioid exposure lead to a decrease the cell area in the DHA
421 group ($F_{(3, 18)} = 6.760$, $p=0.003$, one-way ANOVA, **Figure 5C**). Next, we measured the mean grey value or
422 average pixel density of the CD68+ cells. This analysis revealed that the DHA group had a significant
423 decrease in mean density compared to the control diet group, regardless of opioid exposure ($F_{(3, 18)} = 83.17$,
424 $p<0.001$ in each diet group) (**Figure 5D**). However, there was no effect of opioid self-administration in either

diet group (p=0.4 in each diet group) (**Figure 5D**). We then measured the integrated density of CD68 immunolabeling by taking the sum of the pixels in the standardized selected area within the dorsal striatum. This parameter was similarly altered by diet ($F_{(3, 18)} = 38.42$, $p<0.001$) with the DHA group showing a significant decrease in integrated density, but there was no effect of opioid exposure (p=0.95 in control and p<0.9 in DHA groups) (**Figure 5E**).

4. Discussion

We have previously shown that a PUFA-enriched diet alters the behavioral and cellular adaptations to non-contingent chronic morphine exposure [43]. In this study, we examined how this same dietary regimen altered contingent opioid use. Our novel mouse model behavior paradigm enabled us to assess the effect of volitional opioid exposure across a profile of opioid use that is arguably closer to the human condition. In doing so, we generated a profile of opioid use that differs from most IVSA studies published to date in that we focused on the drug-seeking behaviors during a drug use cycle – of extinction, reinstatement, and another period of extinction – to model the phases of abstinence and relapse typical of opioid exposure. Interestingly, we found that oxycodone IVSA established under a short-access FR1 schedule does not result in an increase in the number of active lever presses or reinforcers gained above that of mice receiving saline during the initial maintenance phase, possibly due to an inverted U-dose effect often seen in such studies [58] However, during an initial extinction period, when the drug is no longer available but all drug-associated cues are present, there is a sharp increase in drug-seeking behaviors irrespective of diet during this first extinction. This drug-seeking declines over subsequent days to a level that is indistinguishable from when the drug is again delivered during the reinstatement phase. A second extinction period results in the same increase in drug-seeking in the control group but now the mice on the DHA diet fail to show this sharp increase in drug-seeking behavior which is indistinguishable from that of mice receiving the saline reinforcer.

Our previous study shows how this DHA enriched diet reduces the anxiogenic behavioral profile induced by morphine and striatal glutamatergic signaling [43]. In this study we investigated how this diet could modulate drug seeking behaviors and the gut microbiome, knowing that there is microbiome-brain axis that modulates anxiety and depression (review; [59]). We show that the addition of DHA altered the gut microbiome during opioid exposure and withdrawal, resulting in increased species richness, phylogenetic diversity, and evenness compared to controls. Since dysbiosis (i.e. disease-associated perturbation of the microbiome) is often associated with lower species richness and diversity, DHA supplementation may protect against adverse phenotypes mediated by the microbiome such as anxiety-like behavior. This effect is similar to previously published studies examining the role of DHA on microbiome-brain axis. For example, Robertson *et al.* showed that DHA supplementation was associated with enhanced cognition in C57BL/6 mice and an overabundance of *Bifidobacterium* [60]. A second study found that DHA-induced enrichment of *Allobaculum* was correlated with reduction of anxiety-like behavior (PMID 27621225). Confirming these prior studies, we show that DHA supplementation was associated with an increase in *Bifidobacterium* and *Allobaculum*. While the comparison of microbial composition before and after opioid introduction was inconclusive, we do demonstrate a significant reduction in *Akkermansia* and *Bifidobacterium* with opioid withdrawal, we do demonstrate a significant reduction in *Akkermansia* and *Bifidobacterium* with opioid withdrawal. Interestingly, the addition of DHA during the extinction phase dampened the reduction of *Bifidobacterium* as compared to control. Prior research has shown that *Bifidobacterium* may increase the bioavailability of opioids by deconjugating glucuronide in the gut lumen [61]. By having a higher level of deconjugating bacteria, DHA may ameliorate the effect of opioid extinction as compared to control. However, the effect of DHA on the IVSA profile during the second extinction phase suggests that the reduction in opioid-seeking is not only the result of increased opioid bio-availability. One

possibility is that DHA-induced *Bifidobacterium* and *Lactobacillus* reduce opioid-seeking behavior through microbiome-brain signaling, consistent with literature demonstrating anxiolytic properties of specific strains within these genera [20, 62]. DHA may also work by suppressing bacteria such as *Akkermansia* that can induce anxiety-like behavior [63].

The underlying mechanism causing the increase in drug seeking behaviors during the extinction cycle is not completely understood and therefore it is also unclear how the DHA enriched diet led to a decrease in these behaviors. Intuitively, it is assumed that the opioid conditioned response is to the positive reinforcement associated with drug use, however, negative reinforcement mechanisms can drive the compulsivity of drug addiction and relapse. The negative emotional state that is experienced during drug abstinence following chronic opioid use and reinforcers paired with drug withdrawal have been shown to lead to drug seeking behaviors [64]. Studies in mice and humans suggest that an elevated level of stress and anxiety increase the probability of relapse and that exposure to stressors reliably reinstates drug seeking behaviors even after prolonged drug-free periods. This negative emotional state and the associated negative reinforcements may be the driving influence in our study that leads the control mice to increase their drug seeking behaviors during the extinction phases of this behavior paradigm. The reduction in drug seeking behaviors due to the DHA supplementation corresponded to gut microbiome changes during opioid maintenance and withdrawal. The DHA supplementation could be playing a protective role in the gut-microbiome by mitigating the microbiome changes (i.e., the reduction in *Bifidobacterium*) observed due to opioid withdrawal during the extinction phase. The DHA enriched diet also alleviated the anxiety-associated behaviors in the light/dark test, which was induced by the opioid use and subsequent withdrawal during the extinction period. There is a strong possibility that the effect of DHA supplementation seen in the gut microbiome offset the increased levels of anxiety during forced withdrawal period caused by extinction.

Intermittent noncontingent but not continuous morphine has been shown to alter the gut microbiome and to induce neuro-inflammation. This can be offset by restoring the microbiome to control levels [65]. In light of our findings that the DHA diet altered the gut microbiome and offset some of the opioid-induced changes seen during the second extinction phase, we hypothesized that the PUFA-enriched diet would reduce microglial activation induced by oxycodone self-administration. Assessing the size of the microglia, identified by the presence of CD68 labeling and shape and intensity of IBA-1 label CD68 cells, we found that this diet reduced the basal activation state of microglia, shown as an increase in size and reduction in the number of cells and intensity of label. However, contrary to our hypothesis, there was no effect of oxycodone self-administration on microglial activation. This may be a result of the low, continuous levels and relatively short access (2 h) of opioid self-administered across the 27 days of the experiment, a regimen that is similar to continuous opioid exposure [65] that may not induce measurable neuro-inflammation. The low sample number of this experiment also warrants caution in furthering our interpretation of this dataset.

This study outlines a profile of opioid self-administration of an initial maintenance phase followed by periods of abstinence, when drug-seeking becomes pronounced, and may imitate relapse. It is this heightened period of drug-seeking that can be reduced by supplemental DHA which, we also show, reduces anxiety. We propose that such periods of opioid exposure and subsequent withdrawal generate a long-term pathological state of anxiety that leads to the high rate of relapse associated with opioid use and that this may be offset by DHA supplementation. As DHA has beneficial effects on the gut microbiome, this PUFA may offset the effect of opioids on the gut-brain axis. The evidence for this interaction between the gut microbiome and neuropathology continues to grow; we propose that this may be the link by which

DHA offsets the cellular and behavioral effects of opioids as shown in this, and our previous work. Additional research does need to be completed in order to understand if affective-like behaviors and long-term drug seeking behaviors could be treated by normalizing the intestinal environment. Whichever the mechanism involved, our findings do suggest that this supplemental dietary intervention could form part of a treatment protocol for opioid use disorder.

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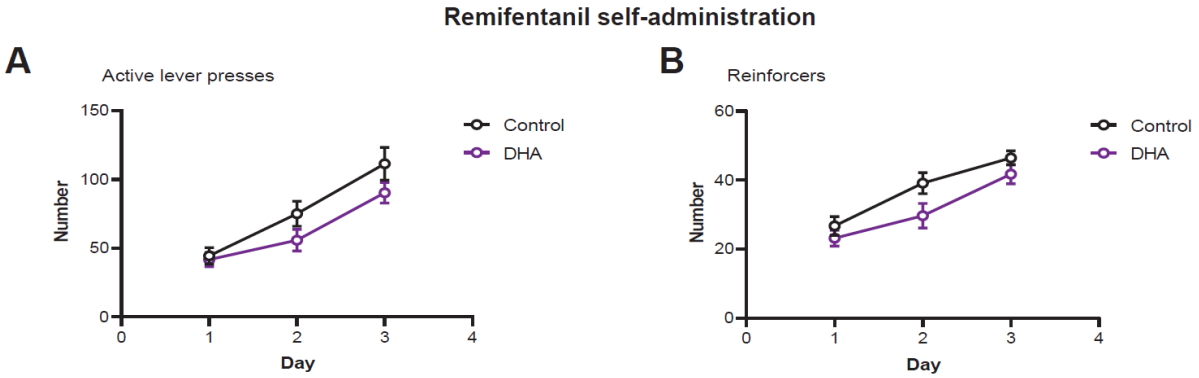
Conflict of Interest; NM is an employee of ZS, but this work represents his personal views and contribution. ZS did not participate or sponsor this work in any form. All other co-authors declare no conflict of interest.

Supplementary Information



Supplemental Figure 1. The acquisition of remifentanil self-administration. (A) There was no effect of diet (control diet shown in black, DHA diet shown in purple) in the initial acquisition of opioid self-administration using remifentanil as the RNFS when observing (A) AL presses (p=0.3) or **(B)** RNFS earned (p=0.4).

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682 **Supplemental Figure 1. The acquisition of remifentanil self-administration. (A)** There was no effect of
683 diet (control diet shown in black, DHA diet shown in purple) in the initial acquisition of opioid self-
684 administration using remifentanil as the RNFS when observing (A) AL presses (p=0.3) or **(B)** RNFS earned
685 (p=0.4).