UNIVERSITY OF CALIFORNIA, SAN DIEGO

Circulating Triglycerides and The Brain's Reward Circuit

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by

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ABSTRACT OF THE THESIS

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Following a lipid rich meal, circulating triglyceride levels increase and some level of reward is experienced. We suspect that a dietary triglyceride processing enzyme known as lipoprotein lipase (LPL) is the sensor involved in triglyceride sensing in the mesolimbic brain. We also believe that circulating triglycerides in the brain increase activity of the reward circuit, signaling positive reinforcement and in cases of overexposure, promoting neuroadaptations similar to those seen in individuals addicted to substances of abuse. Using fluorescent in-situ hybridization we found that LPL's expression was specific to specific cell types within mesolimbic circuitry, namely dopaminergic and medium spiny neurons. By chronically catheterizing the carotid artery of mice, we were able to directly infuse triglycerides to the brain via catheter in order to emulate post-meal neurophysiology. We performed lipid profile analyses of mice that had received brain triglyceride delivery (BTGD) and found significant changes in striatal glycerolipid, free fatty acid, and eicosanoid species, validating our approach to examining

post-meal neurophysiology and pointing to activation of the pro-inflammatory lipoxygenase (LOX) pathway. Using immunohistochemistry following BTGD, we found that circulating triglycerides may upregulate markers of neural activity in mesolimbic and basal ganglia circuitry. Lastly, we assayed the effects that circulating triglycerides may have on anxiety, and found mice that received BTGD exhibited a more anxious behavioral phenotype. We therefore have further reason to believe in the ability of palatable foods to act as an addictive substance, and in the role of LPL as a lipid sensor in the mesolimbic brain.

Introduction

Diabetes, obesity, and what has come to be known as metabolic syndrome all impart strong individual and societal costs. It was reported that in 2014, over 1.9 billion adults worldwide were overweight, where 600 million of them were obese. It was also reported that 422 million adults were living with diabetes globally; meaning the age-standardized prevalence of diabetes has staggeringly doubled since 1980 (World Health Organization, 2016). While it is a common misconception to believe that these ailments are born *solely* out of personal disposition, they are somewhat preventable and are in part caused by unrestricted feeding, physical inactivity, and a preference for strongly rewarding hyperpalatable (high fat, high sugar) foods, which are ubiquitous in many modern societies. Diabetes and obesity are also fueled by individual issues regulating consumption – issues that we believe are partly rooted in abnormalities of reward circuitry. Both ailments contribute to poor quality of life, health deterioration and comorbidities, and ultimately a reduced lifespan.

Normal post-meal lipid neurophysiology as it relates to our objectives can be simplified to three key steps. Firstly, following a meal triglyceride levels increase, and remain elevated in obese individuals (Ruge et al., 2009). Next, triglycerides enter the brain, and in areas known to express lipases (Wang & Eckel, 2012). Lastly, some level of reward is experienced; interestingly the degree of reward has been shown to correlate with how satisfying the palatable food was upon consumption (Bassareo & Di Chiara, 1999; Small, Jones-Gotman, & Dagher, 2003). While the occurrence of these events have been studied and documented, the mechanisms by which triglycerides enter the brain and the mechanisms driving their rewarding effects on reward have yet to be elucidated.

The reward circuitry of the brain includes the Ventral tegmental area (VTA) and its projections. The VTA is a brain structure that signals reward primarily by releasing dopamine from dopaminergic neurons that project to other areas of the brain.

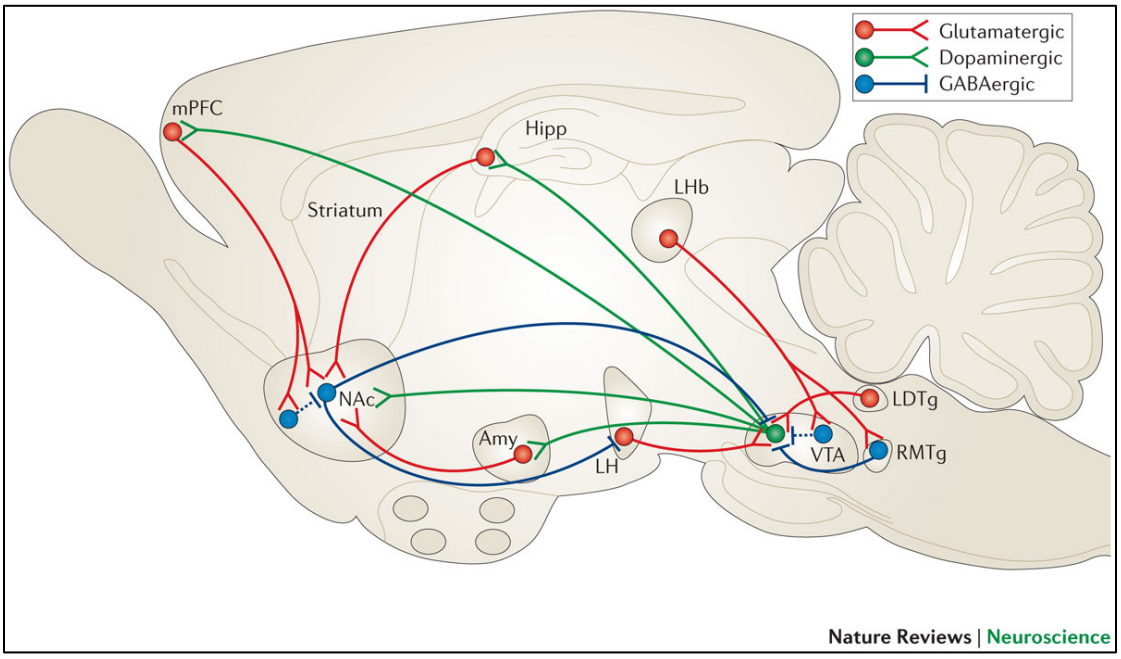


Figure 1. Brain reward circuits in the mouse. The mouse brain's reward circuitry is fairly analogous to that of a human, and is similar in complexity (Russo and Nestler, 2014).

Indeed the reward circuitry of the brain is quite complex, and the VTA projects to many brain structures including the prefrontal cortex, striatum (Caudate putamen and nucleus accumbens), and amygdala; however typically referred to as "the reward pathway" is the dopaminergic input to the nucleus accumbens (Nestler & Carlezon, 2006; Russo & Nestler, 2014). This input is received by GABAergic medium spiny neurons (MSNs) that express dopamine receptor types 1,2 (DRD1, DRD2) – G_s and G_i coupled receptors respectively (Figure 1).

Palatable foods have many characteristics in common with drugs of abuse, most notably that they both act on the brain's reward circuit, can serve as positive reinforcers,

and can yield irregular neuroadaptations in the reward circuitry of the brain that facilitate addictive behavioral phenotypes (Bassareo & Di Chiara, 1999; Cansell et al., 2014; Johnson & Kenny, 2010; Kenny, 2011; Nestler, 2005; Small et al., 2003). Reward circuits of addicted brains have several typical hallmarks, including decreased basal dopamine and glutamate release, however there are also specific irregularities that drive forward the compulsive and unrelenting reward seeking behaviors common in addicts (Nestler, 2005). For instance, both drugs of abuse and high fat diets have been shown to increase striatal expression of the transcription factor, delta Fos B – increasing motivational or compulsive reward seeking behaviors (Kenny, 2011). Additionally, it has been shown that extended access to palatable foods manifests reward threshold elevations in rats, and that DRD2 impairment increases vulnerability and expedites those abnormalities – abnormalities that are similar to those found in addicts (Johnson & Kenny, 2010). Several other studies have found reward circuit irregularities among obese subjects (those who are likely to have issues regulating consumption) that are also common to addicts. Notably, it has been shown that individuals suffering from obesity displayed decreased DRD2 receptor signaling and less reward circuit activation – similar to the reduced dopamine release triggered by drug consumption observed in addicted individuals (Kenny, 2011; Volkow, Wang, Fowler, Tomasi, & Baler, 2011). These individuals also exhibited decreased baseline activity of the prefrontal cortex – a brain region known to play a role in executive function and inhibitory control. Once again, this decrease in baseline activity of the prefrontal cortex is also seen in both addicted and obese individuals. Given the striking similarities in reward circuit abnormalities that occur among obese individuals and those addicted to known substances of abuse,

unraveling the underlying circuit, cell, and molecular level components of *how* palatable food may act as an addictive substance is another front on which we can move toward alleviating the burdens imposed by metabolic disorders.

The brain and specifically the reward circuit express the triglyceride processing enzyme Lipoprotein Lipase (LPL), which is suspected to play a role in the sensing of lipids at the level of reward and motivation. As a lipase, LPL hydrolyzes triglycerides into fatty acids, which are precursors to bioactive signaling molecules that may be responsible for the behaviors mediated by brain triglyceride action. While understudied, what has been found thus far within the field of dietary triglyceride neurophysiology is fascinating. Specifically, the Luquet lab (Cansell et al., 2014) has discovered profound effects on behavior caused by detection of triglycerides at the level of the brain. The Luquet lab has established a novel, innovative procedure to infuse dietary triglycerides directly through the carotid artery and toward the brain through their normal route of delivery. The catheter is threaded out through the nape of the mouse allowing infusion in awake and behaving animals. It is vital to note that this method infuses a 20% lipid emulsion, Intralipid, consisting primarily of vegetable oil triglycerides, and that the rates of infusion are very low (0.1 – 0.5 ul/min) so as to not alter peripheral blood chemistry (Cansell et al., 2014). In essence, this method has been titrated to emulate the brain's environment following a high fat meal in isolation from peripheral metabolic physiology (e.g avoiding insulin, leptin, ghrelin release).

By use of this method the Luquet lab discovered that BTGD evokes several behavioral changes consistent with homeostatic feedback mechanisms.

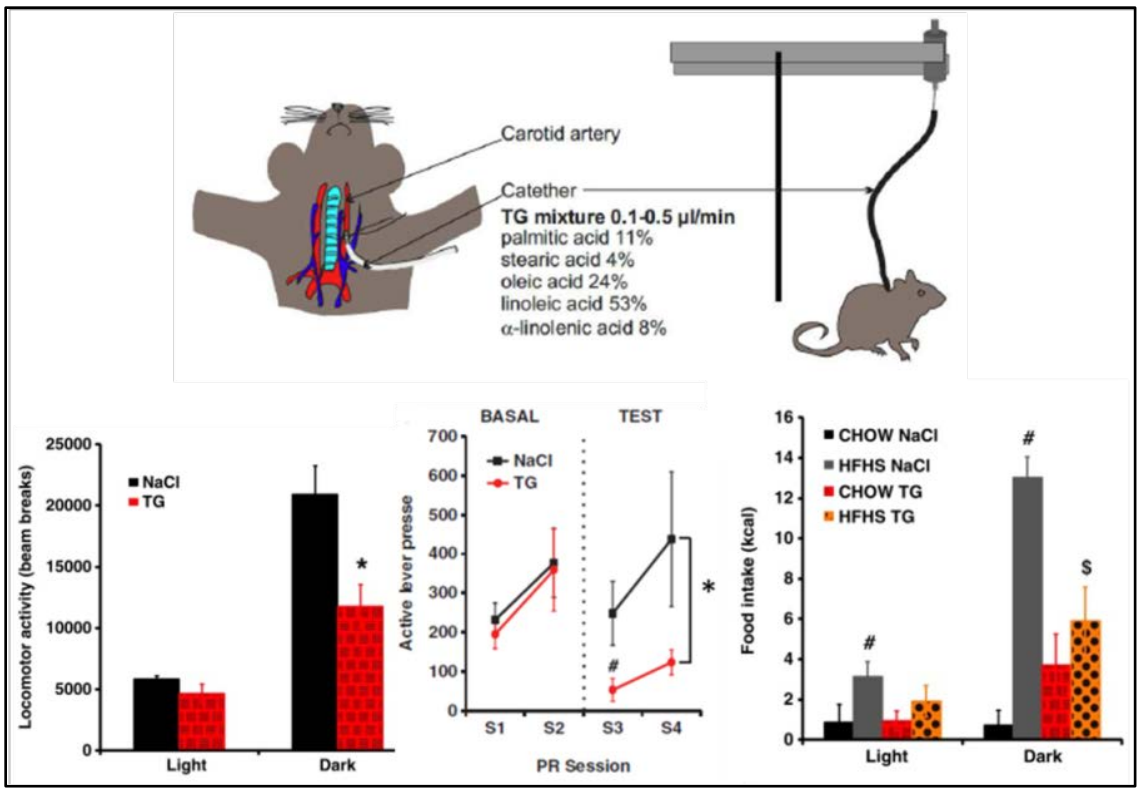


Figure 2. Circulating triglycerides in the brain affect behavior.
Clockwise from the top: Using a novel method to deliver dietary triglycerides directly to the brain in awake and behaving mice by chronically catheterizing the mouse carotid artery, it has been shown that circulating triglycerides in the brain reduce preference for palatable food, motivation to engage in food seeking, and nocturnal locomotor activity, all of which are behavior's that are in part determined by mesolimbic function (Data from Cansell et al., 2014).

Specifically, they noticed that mice have reductions in their innate preference for palatable foods, reward seeking behaviors, and locomotor activity (Figure 2).Taken together, reward seeking behaviors and an innate preference for rewarding palatable foods lead to the continuous influx of circulating triglycerides in the brain provided by consumption of said foods. The BTGD evoked feedback mechanism of reducing food-seeking behaviors serves as way to reduce the amount of circulating triglycerides in the brain.

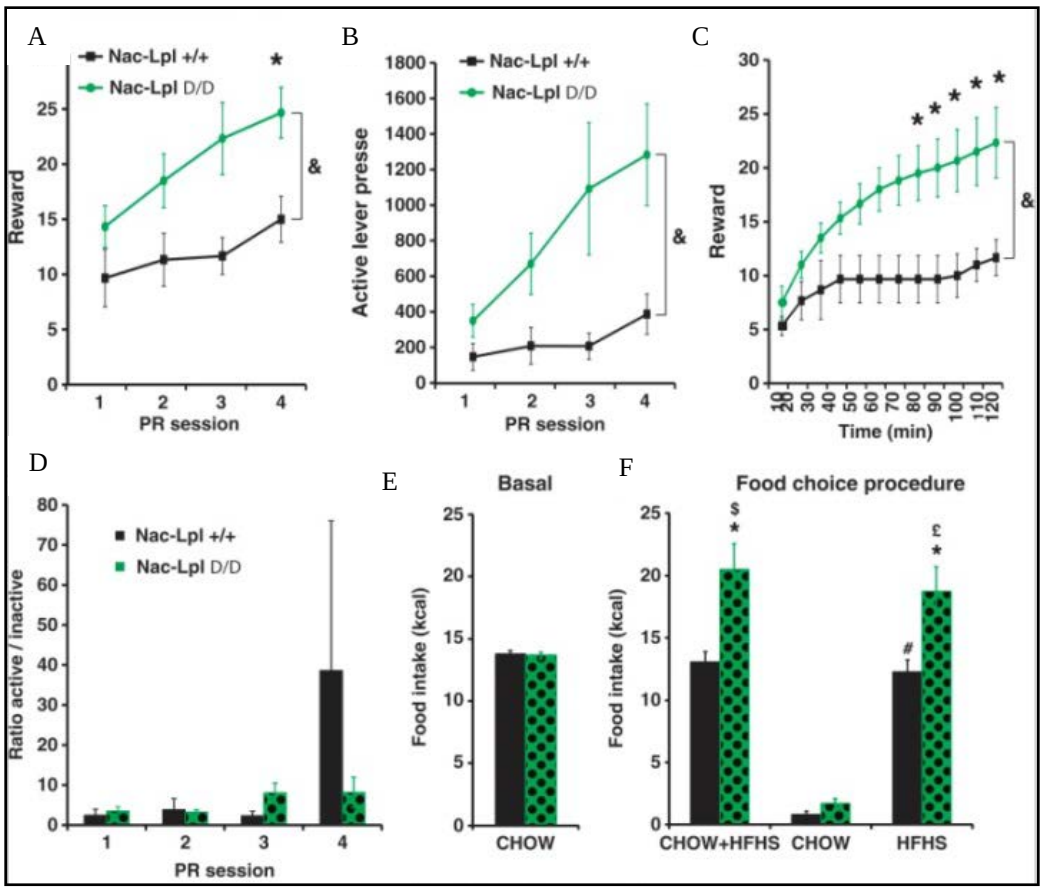


Figure 3. Nucleus Accumbens LPL knockdown reverses normal triglyceride response. LPL knockdown mice exhibited increased reward seeking behaviors (A, B, C, D) and returned to their innate preference for palatable food (E,F) compared to their wildtype counterparts (Data from Cansell et al., 2014).

Strikingly, targeted LPL knockdown in the nucleus accumbens produces opposite behavioral effects, implicating it as a key factor in the detection of circulating triglycerides within the mesolimbic circuitry of the brain (Figure 3). As a lipase, LPL mediates triglyceride hydrolysis, suggesting that the processing of triglycerides is fundamental to the observed effects of their infusion.

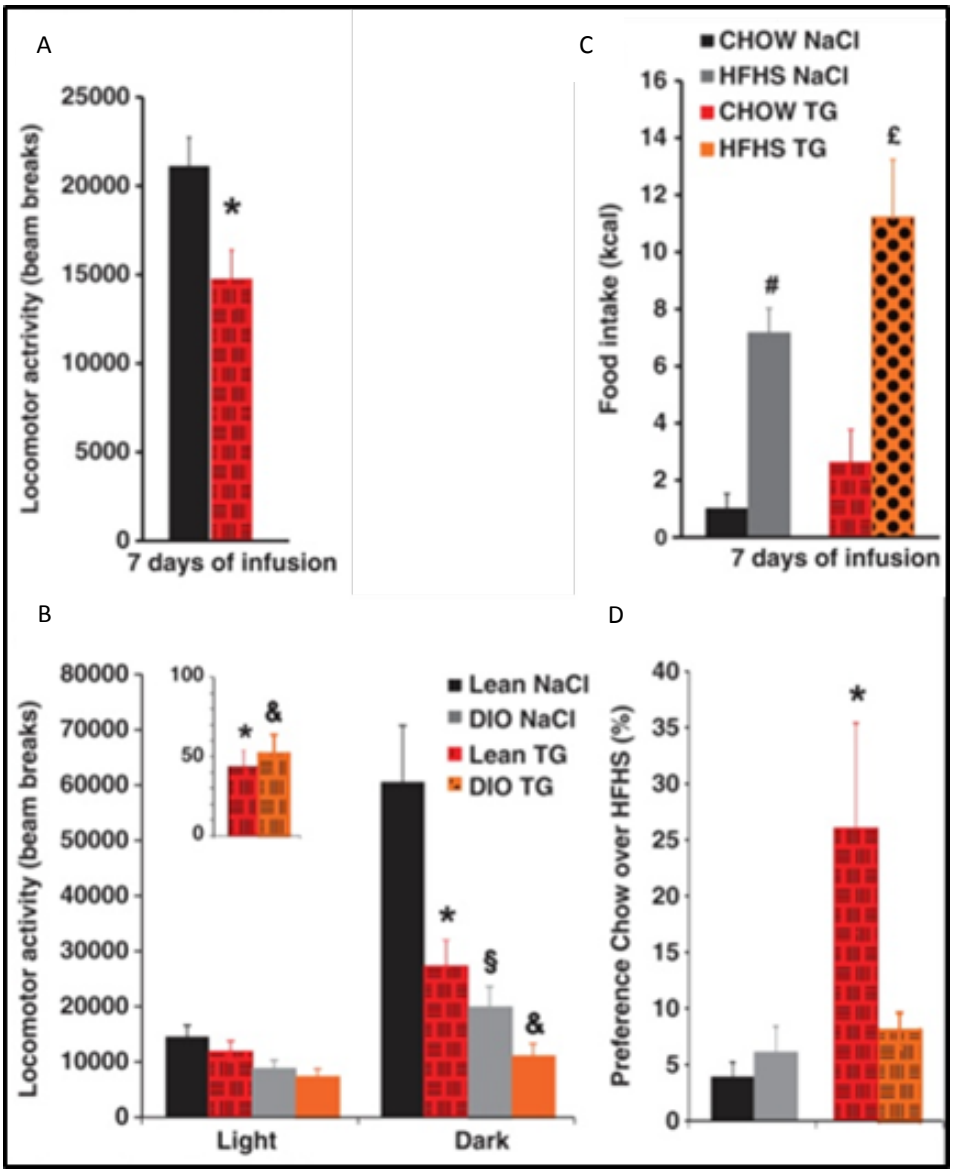


Figure 4. Prolonged triglyceride exposure leads to irregularities in triglyceride detection and response in the brain.
In cases of hypertriglyceridemia, either incurred by 7 days of infusion or diet induced obesity, BTGD ceased to affect food preference (C,D), allowing the animals to return to their innate preference for palatable foods, yet continued to reduce locomotor activity (A,B) (Data from Cansell et al., 2014).

In cases of hypertriglyceridemia, triglycerides may continue to reach the brain and be detected, however some of the initial behavioral feedback mechanisms disappear with sustained delivery. For instance, preference for palatable food returns, while reduced motor activity persists. This suggests that in individuals with hypertriglyceridemia there

arise some neuroadaptations that may further contribute to metabolic dysfunction by altering the normal behaviors evoked by triglyceride sensing in the brain, namely a reduced preference for palatable foods.

Despite the link between infusing dietary triglycerides in the brain, the evoked behaviors, and LPL - the actual molecular and cellular mechanisms or signaling cascades underlying the detection and method of action of lipids has yet to be uncovered. It was our aim to study these mechanisms, and to further investigate the role that LPL may play as a sensor of circulating triglycerides in the brain's reward circuitry.

Materials and Methods

-Mice

C57Bl/6J Jackson Laboratory stock mice were used for all experiments. All procedures and animal care were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of California, San Diego. Mice were housed on a 12:12 hour light-dark cycle, in a temperature and humidity controlled vivarium, and allowed food and water ad libitum. Mice were group housed, and switched to individual cages post-operatively. All mice used were at least 6 weeks of age. When paired, animals were sex and age matched.

-Fluorescent in-situ hybridization.

In order to delineate the expression patterns of LPL, dopaminergic neurons, and dopamine receptors within the mesolimbic circuitry of the brain, the RNAscope, a commercialized in-situ hybridization assay was used. Mice were euthanized, and had their brains immediately extracted and flash frozen in isopentane. Brains were sectioned at 20 um on a Leica cryostat, and sections were probed for gene expression as per the protocols provided by Advanced Cell Diagnostics. TH and LPL expression were assayed in the VTA; DRD1, DRD2 and LPL expression were assayed in the both the CPu and NAc. Z-stacked images were captured with a Zeiss AxioObserver fluorescent microscope, Zeiss Apotome 2.0, and through a 63x objective lens. Three age and sex matched mice had their VTA, CPu, and NAc assayed. Each brain structure had 3 bregma points sampled. VTA sections had 3 images taken from the lateral VTA, and both CPu as well as the NAc sections had 4 images taken each. Counts were added per bregma point, and per structure so as to have a representative sample. Cells that exhibited at least 4

puncta, or RNA molecules in addition to DAPI were counted as expressing the respective gene.

-Surgeries

By chronically catheterizing the carotid artery of mice, we were able to infuse triglycerides directly to the brain via catheter in order to emulate circulating dietary triglyceride neurophysiology.

Once anesthetized under isofluorane (2%), the catheterizations were performed. They consisted of an incision at the throat, insertion and securing of a catheter into the carotid artery, threading the catheter through the nape of the neck, and suturing the incision site closed. Mice were given intraperitoneal injections of Carprofen at 5ug/kg body weight as an analgesic, and povidone-iodine as an antiseptic following surgery.

-Brain Triglyceride Delivery (BTGD).

Infusions were performed using a programmable dual syringe pump (Braintree Scientific BS8000) that connects a syringe to the respective mouse's catheter. Infusions were performed in paired, sex and age matched, awake, and behaving mice that either received a 20% Intralipid lipid emulsion consisting of soybean oil, a triglyceride, (Sigma 68890-65-3) or saline. Rates of infusion never surpassed 0.3 ul/minute. Importantly, it has been shown by the creators of this technique that these low rates of infusion do not alter the peripheral blood chemistry of the animal, allowing the technique to remain a physiologically sound model of lipid sensing strictly in the brain (Cansell et al., 2014).

-Lipidomics.

Seven pairs of mice were infused at 0.2 ul/min for 4 hours with either saline or triglycerides. Immediately following infusion, mice were euthanized, and had their

striatal (CPu and NAc) brain tissues rapidly extracted and frozen on dry ice. Lipid and protein analyses were performed at the UCSD Lipidomics Core (Quehenberger, Armando, & Dennis, 2011; Quehenberger & Dennis, 2011). Relative abundances of individual species per sample were normalized to protein concentration per sample.

-c-Fos Induction and Immunohistochemistry.

To investigate changes in neural activity in response to circulating triglycerides, we performed tyrosine hydroxylase and c-Fos immunohistochemistry on brain areas implicated in the reward circuit. Mice were catheterized and allowed one week for recovery and return to normal weight. Once recovered, mice were connected to the infusion pump and allowed to habituate individually for 3 hours in a clear red acrylic cage. Once habituated paired mice were infused at a rate of 0.3 ul/min for 2 hours, staggered for 20 minutes in between mice.

Mice were anesthetized with pentobarbital (Euthasol), and transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered-saline (PBS). Brains were extracted, post-fixed for 24 hours in 4% PFA, cryoprotected 30% sucrose for 72 hours, and flash frozen in isopentane. Brains were then sectioned at 30 um on a Leica CM3050s cryostat and preserved in 0.01% sodium azide in PBS. Tyrosine hydroxylase (Pel-Freez P60101-0) and c-Fos (Cell Signaling 2250S) immunohistochemistry were performed on sections of interest. Sections were mounted using Fluoromount-G media (Southern Biotech) containing dapi, and coverslipped.

Tiled and stitched images of entire brain structures were captured using a Zeiss AxioObserver fluorescent microscope, through a 20x objective lens (Example shown in Supplementary figure 1). The following brain regions were surveyed: Prefrontal cortex

(cingulate cortex, prelimbic cortex, and infralimbic cortex), nucleus accumbens (NAc), caudate putamen (CPu), ventral pallidum (VP), globus pallidus external (GPe), sub-thalamic nucleus (STN), substantia nigra reticulata (SNr), and ventral tegmental area (VTA). Each brain region had 4 equidistant bregma points for c-Fos expression (with the exception of the smaller sub-thalamic nucleus, which only had 3). After, c-Fos expression was quantified in brain regions of interest. Only cells that showed both Dapi and c-Fos were counted as c-Fos positive. Tyrosine hydroxylase expressing cells that co-expressed c-Fos and Dapi were also counted.

-Behavior.

Eight pairs of mice were infused at 0.3 ul/min for 1 hour with either saline or intralipid. Mice were then place on an elevated plus maze (EPM) constructed out of white acrylic, and video tracked with Anymaze software for 5 minutes. Mice were placed on the center of the maze and facing an open arm. Ratio of time spent and entries in open and closed arms were analyzed. An arm entry was counted as 80% of the animal's body entering the arm; in order for an exit not to occur, at least 75% of the animal needed to remain in the arm. The maze consisted of two open and closed arms respectively, with like arms facing opposite one another. All arms of the maze were 35 cm long and 5 cm wide, with a neutral middle area of 5 cm by 5 cm. The walls of the closed arms spanned the length of the arm, 35 cm, and were 20 cm high. The EPM itself was 50 cm off the ground.

-Statistics.

Paired, two-tailed, parametric t-tests were used for all statistical analysis. Significant values were those with $P < 0.05$. All error bars represent +/- SEM.

Results

In order to gauge what mechanisms may underlie triglyceride sensing in the brain, and, we delineated the specific expression patterns of LPL in the mesolimbic system. Considering the circuitry that is known to encode the reward associated with foods rich in triglycerides, we suspected that LPL would appear in the presence of mesolimbic cell types, namely either dopaminergic or dopamine receptor expressing neurons.

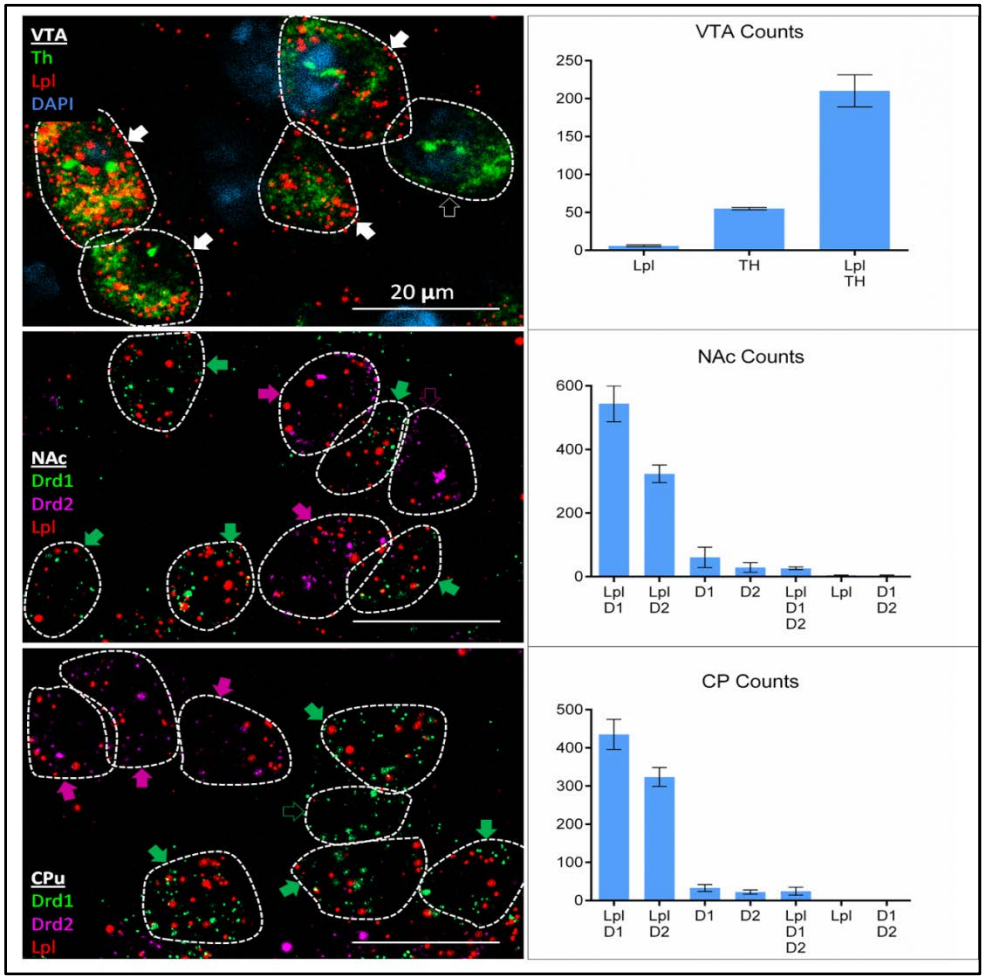


Figure 5. LPL co-localizes with cell types present in the reward circuit.
Indeed we found that LPL strongly co-localizes with TH positive neurons in the VTA and dopamine receptor expressing medium spiny neurons (MSNs) in the striatum. Within these structures, LPL almost never appeared in the absence of either of these cell type markers.

-VTA

Nearly 80% of each animal's TH+ cells in the VTA co-expressed LPL, and LPL alone represented 2% of the cells counted. The remaining cells were TH+ neurons that did not express LPL.

-CPu

Nearly all (>99%) of LPL positive neurons appeared in either a DRD1 or DRD2 MSNs, with 94% and 92% of DRD1 and DRD2 receptor expressing MSNs respectively also expressed LPL.

-NAc

Nearly all (>99%) of LPL positive neurons appeared in either a DRD1 or DRD2 MSNs, with 90% and 93% of DRD1 and DRD2 receptor expressing MSNs respectively also expressed LPL. Images were taken of areas known not to express LPL, TH, DRD1, or DRD2 as negative controls for the probes. Indeed, there was no expression of these genes, and what little signal was found was actively searched for in the region (Supplementary figure 2).

To investigate the molecular events following in the detection of circulating triglycerides within the reward circuit, and any signaling cascades that may be further responsible for the behavioral effects of BTGD, we performed lipidomic analyses on CPu and NAc, both tissues known to express LPL. We assayed glycerolipids (triglycerides and diglycerides), free fatty acids, and eicosanoid lipid profiles.

Table 1A. BTGD results in increased glycerolipid species. Only one species was a diglyceride, 38:5 DAG, while the others were longer chain triglycerides.

A. Glycerolipid	TG (relative intensity/mg protein)	Saline (relative intensity/mg protein)	P value
54:4 TAG	0.1007	0.06899	***0.0141***
60:9 TAG	0.008994	0.005516	***0.0189***
52:4 TAG	0.08414	0.0579	***0.0202***
54:5 TAG	0.07751	0.05881	***0.0287***
38:5 DAG	0.2913	0.2182	***0.0332***
50:2 TAG	0.1594	0.1099	***0.0332***
56:6 TAG	0.2352	0.162	***0.0379***
50:0 TAG	0.4922	0.3527	***0.04***
56:4 TAG	0.03254	0.0229	***0.0459***
52:3 TAG	0.1581	0.1152	***0.048***
60:12 TAG	0.08068	0.05009	***0.0481***
50:3 TAG	0.01944	0.01548	***0.0494***
56:5 TAG	0.06834	0.05221	**0.0533**
52:2 TAG	0.4823	0.3677	**0.0536**
Total TAG	4.655	3.528	**0.0649**
54:1 TAG	0.08915	0.07028	**0.068**
50:1 TAG	0.6237	0.4231	**0.0843**
60:11 TAG	0.02401	0.01862	**0.092**
58:6 TAG	0.01989	0.01411	**0.0938**
48:1 TAG	0.07601	0.06114	**0.0998**

Table 1B. BTGD increased striatal free-fatty acids. We found a significant increase in 18:3 N6, or gamma-linolenic acid (GLA) among the triglyceride treatment group.

B. Free Fatty Acid	TG (pmol/mg protein)	Saline (pmol/mg protein)	P value
18:3 N6	2.143	1.329	***0.0216***
20:3 N9	1.629	1.071	**0.0554**

GLA is a known component of vegetable oils, including soybean oil, which comprises the intralipid infused. Mead acid, or eicosatrienoic acid (20:3 N9) is another polyunsaturated free fatty acid that showed an increase among the triglyceride group. Free fatty acids and glycerolipids are listed in the notation X:Y, where X and Y represent the number of carbons and double bonds on the molecule respectively.

Table 1C. BTGD results in an increase of eicosanoids, pointing to activation of the Lipoxygenase pathway.

C. Eicosanoid	TG (pmol/mg protein)	Saline (pmol/mg protein)	P value
12-HEPE	0.1326	0.08373	***0.0069***
17 HDoHE	3.32	2.637	***0.0082***
14,15 LTC4	0.5084	0.3729	***0.0194***
8,9-EET	1.408	1.176	***0.0296***
HXA3	12.97	8.964	***0.0411***
15-HETrE	0.571	0.4813	***0.0472***
20 HDoHE	5.741	5.106	***0.0476***
12-HETE	15.93	14.85	***0.0476***
16HDoHE	1.927	1.631	**0.052**
15 oxoEDE	0.1504	0.1028	**0.0624**
10 HDoHE	0.9302	0.7425	**0.0894**

Various eicosanoid species were found to be significantly elevated. Specifically, the lipoxygenase (LOX) pathway appears to be activated, hence the elevation of the pro-inflammatory compounds: HXA3, 12-HEPE, 12-HETE, and 14, 15 LTC4. The eicosanoid 17 HDoHE is also a LOX pathway byproduct; however it is a precursor to resolvins, which have strong anti-inflammatory properties. Similarly, 8,9-EET is also known to have anti-inflammatory effects. 15-HETrE is a metabolite of the elevated fatty acid 18:3 N6, while 20 HDoHE, 16 HDoHE, and 10 HDoHE are all auto oxidation products of docosahexaenoic acid (DHA; 22:6(n-3)), a product of 18:3 N3 (alpha-Linolenic acid), GLA's isomer. Interestingly, there were no significant differences in ALA or DHA levels between treatment groups (Supplementary table 1C). All lipid species surveyed are graphed and provided in (Supplementary table 1A-D).

In order to uncover structure specific changes in neural activity may occur following BTGD, we quantified c-Fos expression within the reward circuitry of the brain. We suspected that BTGD would upregulate neural activity in the in the VTA and its subsequent outputs considering the rewarding effects of palatable foods.

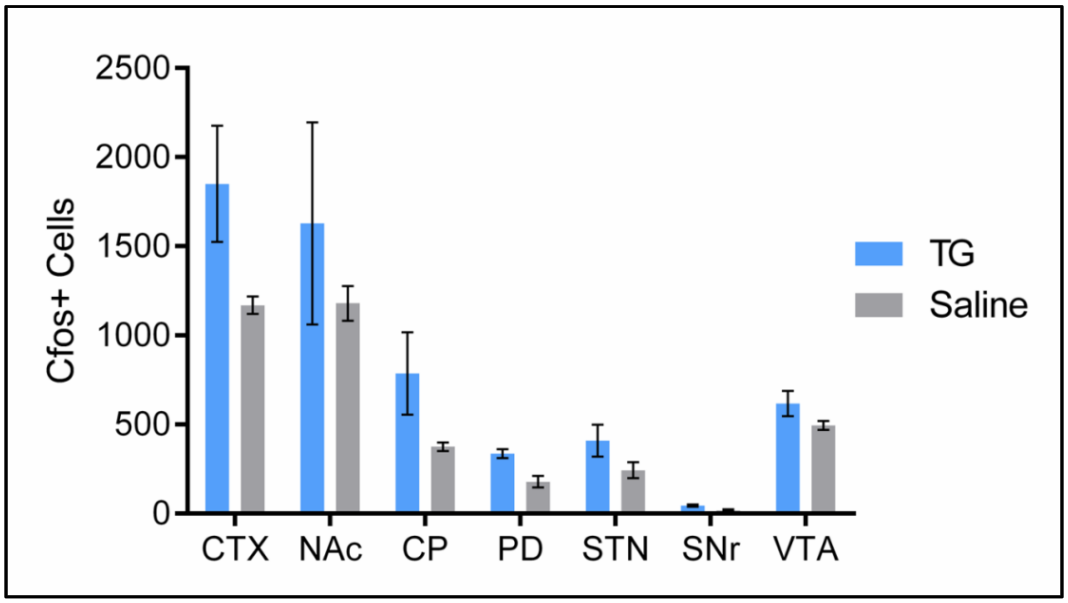


Figure 6. Preliminary data shows BTGD increases neural activity in the reward circuit. We found an increase in c-Fos positive cells in the pre-frontal cortex , NAc, CP, ventral and globus pallidus, subthalamic nucleus, substantia nigra reticulata, and VTA of triglyceride receiving animals (n=2).

There appeared to be some general excitation of the reward circuit following BTGD (Figure 6). Interestingly, there was no difference between the amount or ratio of TH cells that were c-Fos positive in the VTA of the two treatment groups. It is vital to note that this data only represents two pairs of animals, and is statistically insignificant.

We next assessed the concept of comfort feeding at the level of circulating triglycerides in the brain (e.g at a level downstream taste, smell, and digestion). We therefore tested whether circulating triglycerides within the brain have an impact on anxiety. Given the proposed rewarding effects of triglyceride rich foods on mice, we hypothesized that mice receiving BTGD would express a more anxiolytic phenotype, however upon infusion and analysis of 8 pairs of mice, we found evidence for the opposite response (Figure 7).

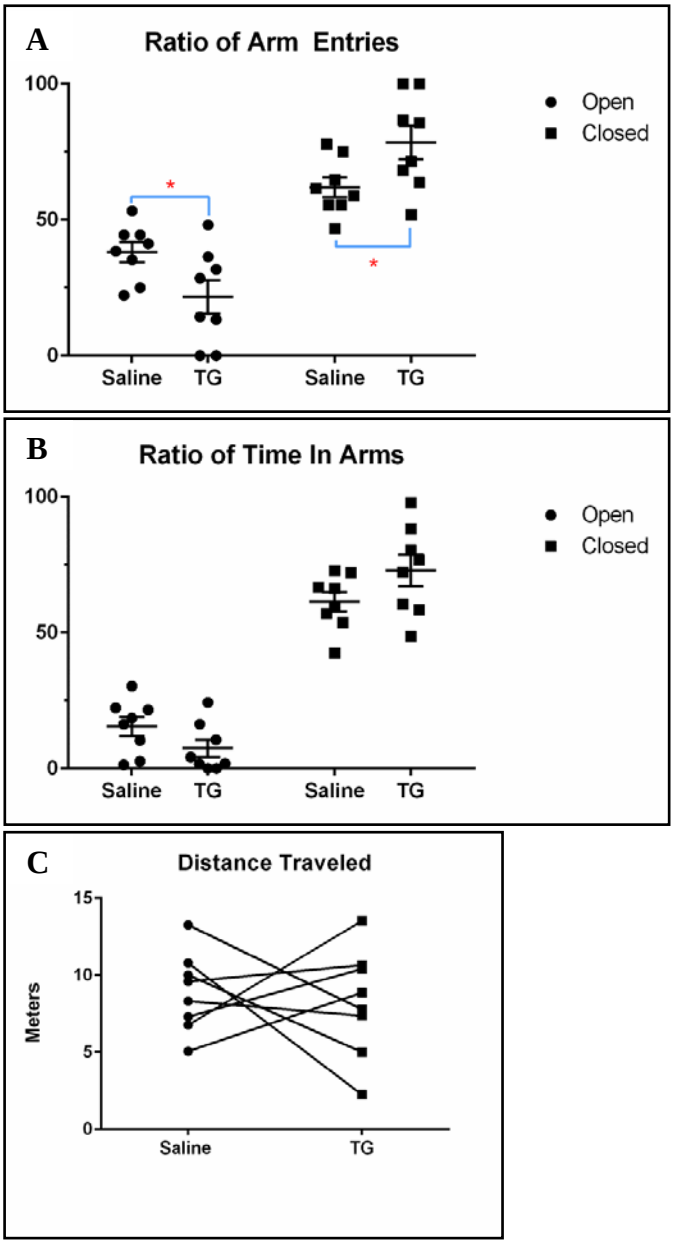


Figure 7A-C: Triglyceride receiving mice showed a more anxious behavioral phenotype. TG receiving mice exhibited a higher ratio of closed arm entries (P = 0.0390) (A) and more time spent in the closed arms of the maze (P = 0.0826) (B). Distance traveled remained unchanged between the two groups (C).

Mice receiving BTGD exhibited an increased ratio of closed arm entries, time spent in the closed arms, accompanied with no detectable change in total distance travelled. The mice otherwise exhibited no visible changes in affect.

Discussion

The data obtained further implicate the reward circuit in responding to circulating dietary triglycerides, and LPL as a sensor in detecting triglycerides at the level of reward and motivation.

Our finding that LPL highly co-expresses in the presence of mesolimbic cell types lends confidence towards the idea that LPL is integral to mesolimbic functions as they relate to dietary lipids. These are functions that are mediated by triglyceride detection and hydrolysis – and we suspect them to be the strong positive reinforcement associated with eating high fat foods in addition to the aforementioned behaviors of reduced food seeking behaviors and reduced preference for palatable foods (Figures 2-4). Coupled with the experimental data wherein NAc specific LPL knockdown reversed BTGD triggered behaviors, we have further reason to suspect LPL as a sensor of dietary triglycerides in the brain (Cansell et al., 2014). In order to delineate LPLs function among various cell types, future experiments should look at targeted impairment and overexpression of LPL in DRD1, 2, and dopaminergic neurons of the mesolimbic system. As an example, recall that DRD2 knockdown in rats facilitates addictive like behaviors in response to extended access to palatable foods; perhaps knockdown of LPL in DRD2 neurons only would produce the same effects, further implicating it as a mediator of triglyceride induced harmful neuroadaptations (Johnson & Kenny, 2010).

Since our physiological model of post-meal lipid neurophysiology appeared to increase activity of the reward circuit – activity known to be a potent source of reinforcement behind many drugs of abuse, we have further reason to test the idea that palatable food can act as an addictive substance as well (Figure 6). The lack of

discrepancy between the proportion of TH+ neurons co-expressing c-Fos between the two groups raises the possibility that the glutamatergic sub-population of neurons in the VTA are activated by BTGD, and they are the cell population that mediates the reward (Hnasko, Hjelmstad, Fields, & Edwards, 2012). Specifically examining cell and molecular adaptations within the reward circuit that may occur in response to prolonged BTGD (and now presumably over activation of the reward circuit) could shed light on how behaviors that further exacerbate metabolic dysfunction may come about, and how they may be treated. Considering the small sample size of animals in the study of neural activity, the experiment should be re-done with a larger cohort of animals in order to bear greater statistical power. Further in-vivo physiology or imaging studies either following or during BTGD would also be fantastic future endeavors.

We also found increases in many triglyceride profiles, especially relative to the small amount of free fatty acid profiles that were elevated. While it may be unsurprising that infusion of triglycerides results in elevation of their lipid profiles, it does validate the approach we are taking in emulating post-meal neurophysiology. These findings further demonstrate that lipids are crossing the blood brain barrier and ending up in the LPL expressing striatum to mediate the observed behavioral effects (Table 1A). The discrepancy between the amount of elevated profiles triglyceride and free fatty acid profiles may be in part due to the time course of infusion. Our experiment totaled four hours from beginning to end; therefore it is possible that only a small fraction of the triglycerides infused were hydrolyzed at the point of tissue extraction. Future lipidomic experiments should vary time courses, lipid doses, and also survey endocannabinoid molecules for any signaling roles that they may play. It would also be interesting to

submit brain regions that are not suspected to play a role in lipid neurophysiology (e.g cerebellum - which we extracted along with the striatum) in order to determine whether lipid profile changes are location specific.

Through our lipidomic analyses we found that our method of BTGD resulted in an increase in GLA, a free fatty acid component of soybean oil, which is the primary lipid present in the lipid emulsion infused (Table 1B). Once again, this finding validates our approach as it demonstrates that the substance we infused, a vegetable oil, reached the brain in the area that we know to mediate the reinforcing properties of foods rich in triglycerides, and was *subsequently hydrolyzed* into its free-fatty acid components. Given that we infused triglycerides and were able to detect increases in components of those triglycerides, namely 18:3 N6, we can further conclude that LPL does indeed act on dietary triglycerides as they are infused in this manner.

The changes we observed in eicosanoids point to the activation of the pro-inflammatory LOX pathway (Table 1C). Interestingly however, several anti-inflammatory compounds were also activated (17 HDoHE, and 8,9-EET), likely as a compensatory mechanism. Several other eicosanoids: 15-HETrE, 20 HDoHE, 16 HDoHE, and 10 HDoHE, all showed significant elevations. Given that 15-HETrE is a byproduct of the fatty acid GLA, this finding gives weight to the possibility that metabolites of specific foods and fats that are consumed can produce specific signaling molecules, and by extension specific signals and behaviors. The remaining elevated eicosanoids, 20,16, and 10 HDoHE are metabolites of docosahexaenoic acid (DHA; 22:6(n-3)), which is a metabolite of alpha-linolenic acid (ALA). In light of no significant difference in ALA or DHA levels of the two groups (Supplementary table 1A), it may be

possible that these eicosanoid changes are too subtle to affect the relative amounts of their upstream components. It is known that high fat diets (regardless of obesity) increase hypothalamic inflammation, and it may be possible that our method of BTGD is responding to the same cue (Waise et al., 2015; Yi, Tschop, Woods, & Hofmann, 2012).

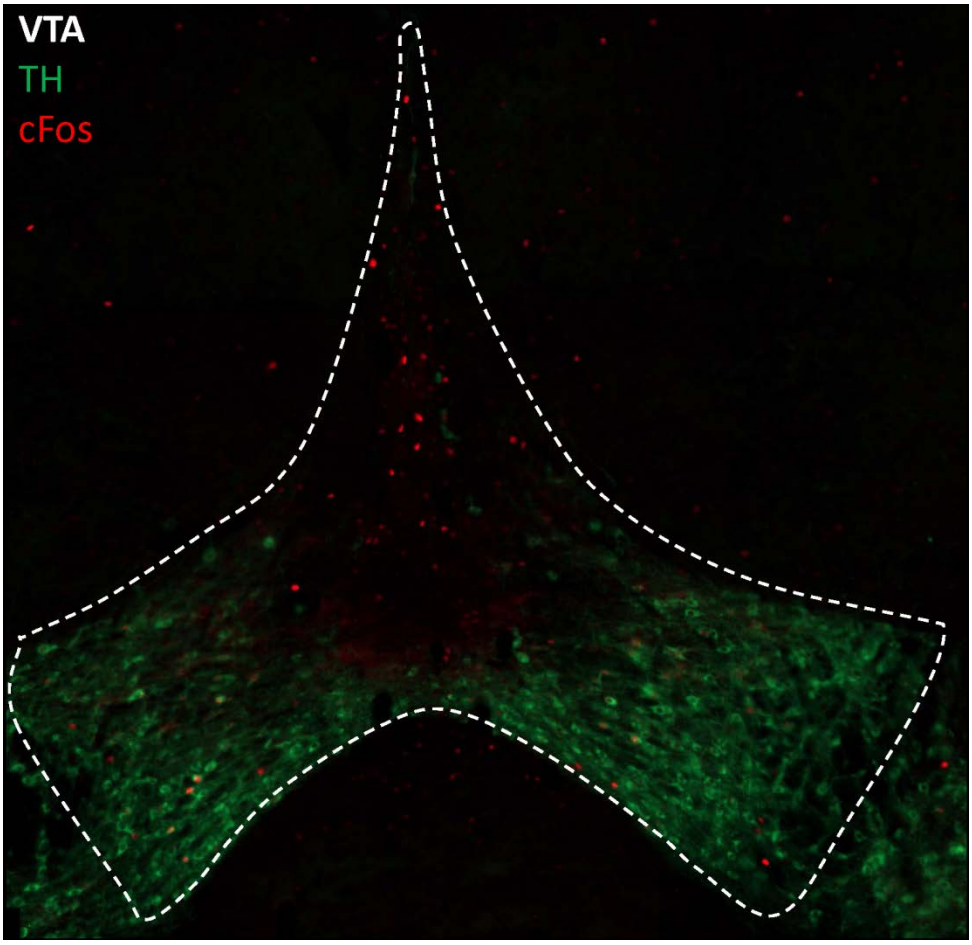
It has also been shown that inflammation triggers brain activation of nuclear factor kappa-beta (NF-KB). NF-KB is a transcription factor that has been shown to cause insulin and leptin resistance, hallmarks of diabetes and obesity respectively. Disruption of brain NF-KB has been shown to have protective effects from obesity in mice fed high fat diets. Taken together these findings present activation of the pro-inflammatory LOX pathway following BTGD as another possible mechanism contributing to the sharp rise in prevalence of diabetes and obesity (Kenny, 2011). Future experiments should examine LOX impairment in the reward circuit and the effect it may have on established behaviors, neuroadaptations, and lipid profiles brought about by BTGD.

There have been several articles with seemingly conflicting data, citing both anxiogenic and anxiolytic behavior following high fat diets (Murphy & Mercer, 2013). In turning toward the literature however it is important to remember that our method of BTGD differs immensely from providing animals with high fat diets. Our method of BTGD prevents changes in peripheral blood chemistry to the brain, and in doing so isolates the lipid response to the brain. Nonetheless, our data demonstrates circulating triglycerides in the brain acting as an anxiogenic agent (Figure 7A-C). It may be possible that the reduced motivation to engage in reward seeking behaviors caused by BTGD also manifests as a reduced motivation to engage in exploratory behaviors, in the sense that exploration is a form of reward seeking in and of itself (Figure 1). There has also been a

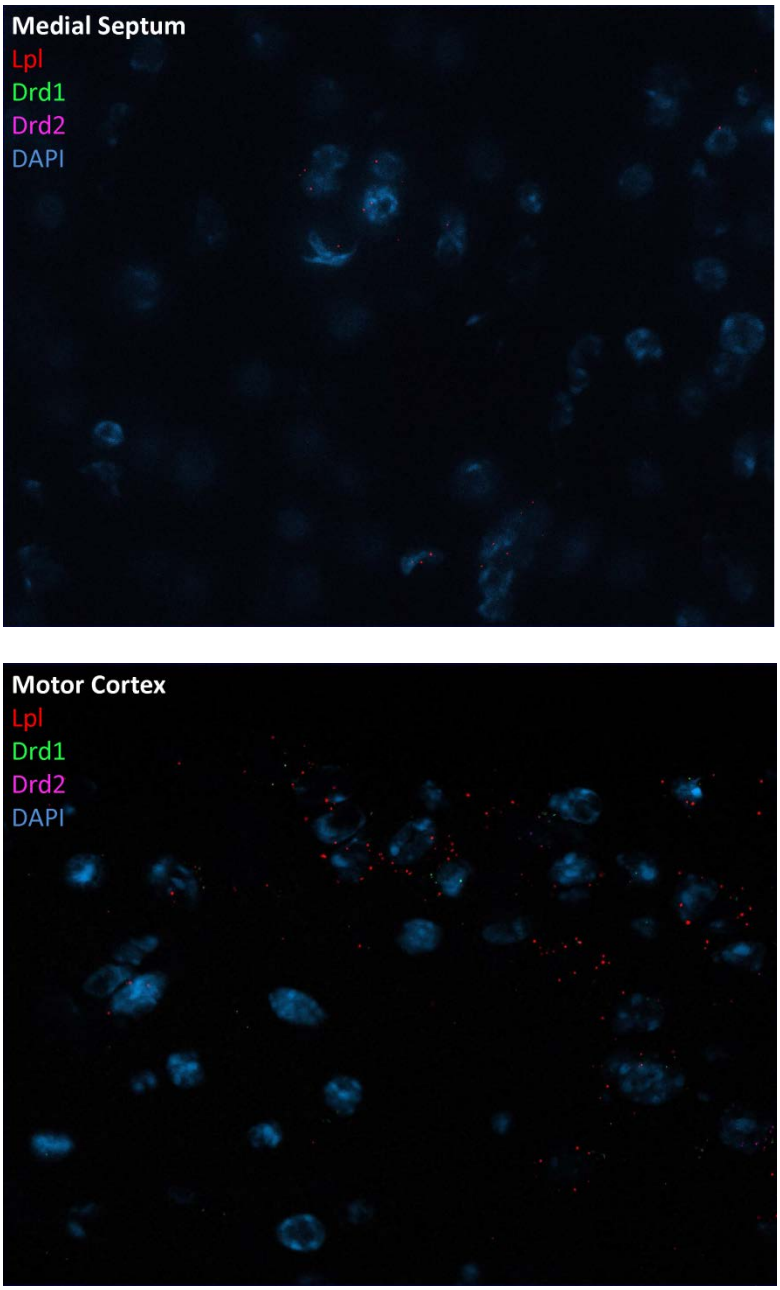
link between inflammation and anxiety established, wherein brain inflammation is seen to be a modulator of anxiety in disease models of multiple sclerosis; it remains possible that the anxiogenic effects of BTGD that we observed were mediated by the LOX pathway (Peruga et al., 2011). In order to validate the effect we saw, future experiment should examine changes in neural activity of the amygdala following BTGD, the effects that impairment of the LOX pathway in brain tissue has on anxiety behaviors following BTGD, and further assays of anxious behaviors including but not limited to open field and light-dark box assays.

In conclusion, we found that BTGD increased triglyceride and free-fatty acid levels, activation of the inflammatory LOX pathway, anxious behavioral phenotypes, and may increase reward circuit activity. We also delineated expression of LPL to dopaminergic and medium spiny neurons in the mesolimbic system. In light of these data as well as shared circuit, cell, and molecular level hallmarks between obese and addicted individuals, we have further reason to believe that detection of triglycerides in the brain may enable *palatable* foods to act as an addictive substance, and in the role of LPL as a lipid sensor in the brain.

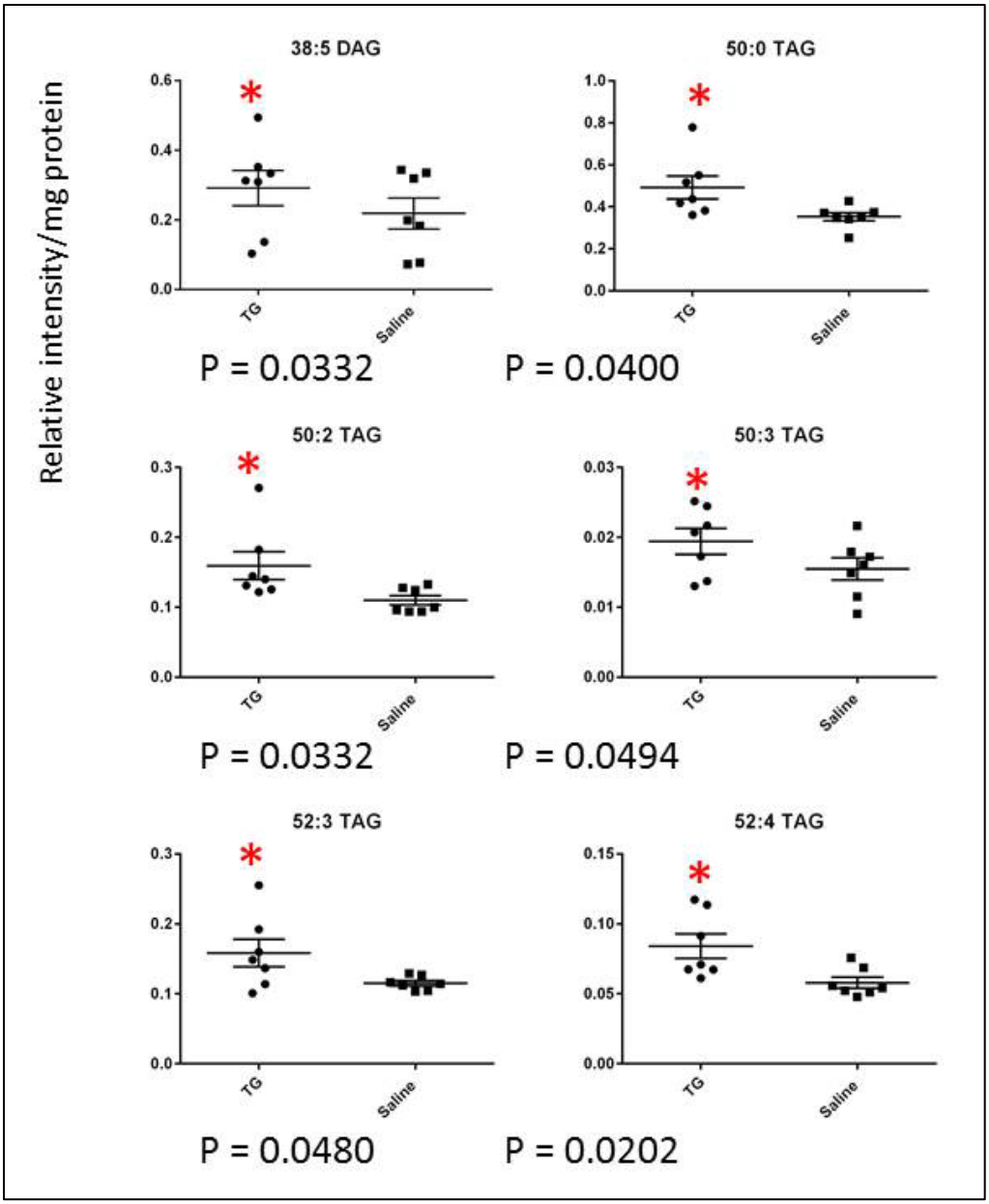
SUPPLEMENTARY FIGURES AND TABLES



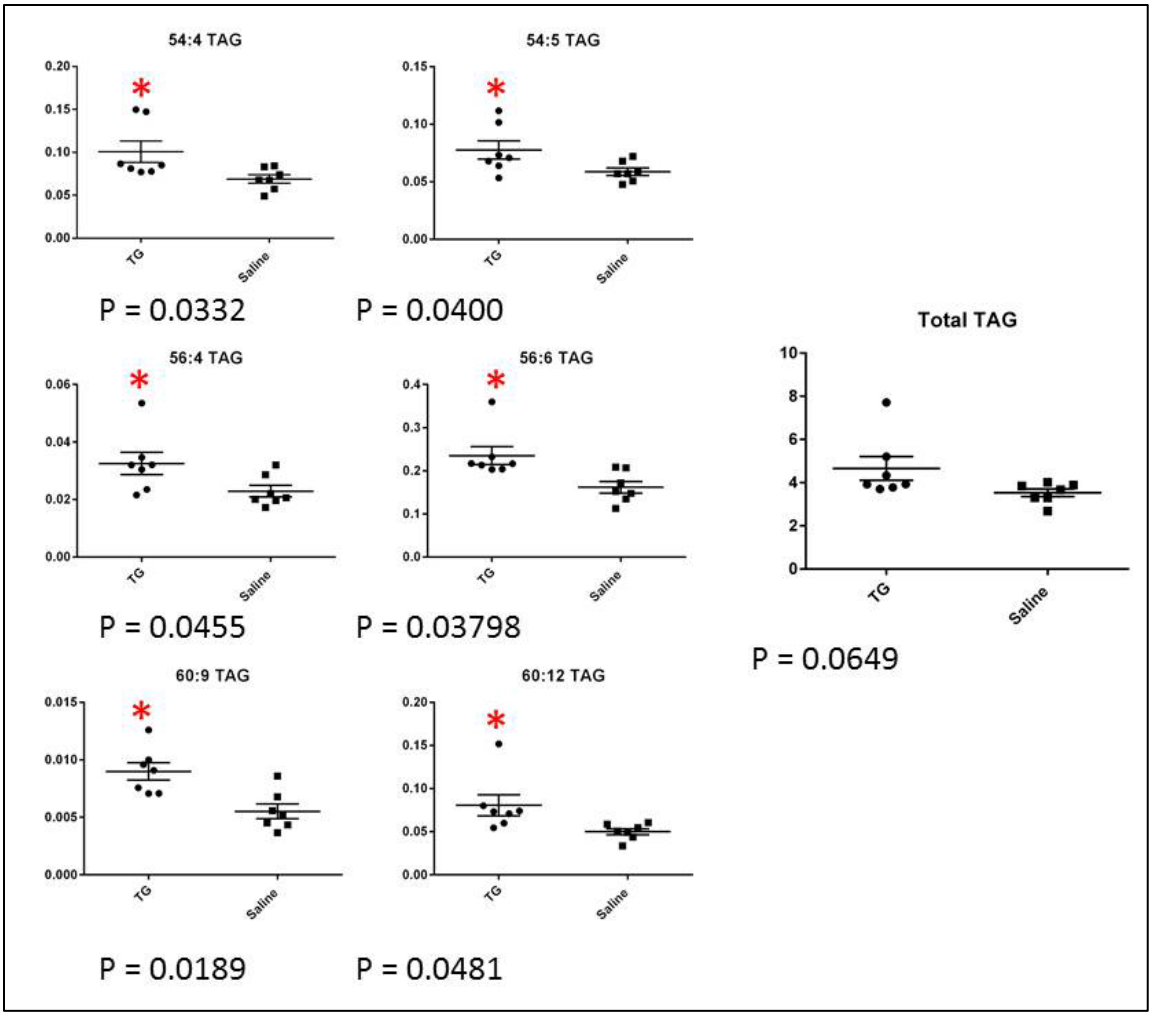
Supplementary Figure 1. Example VTA histology for quantification of cFos+ cells. Only the cells within a given brain structures trace were to be counted.



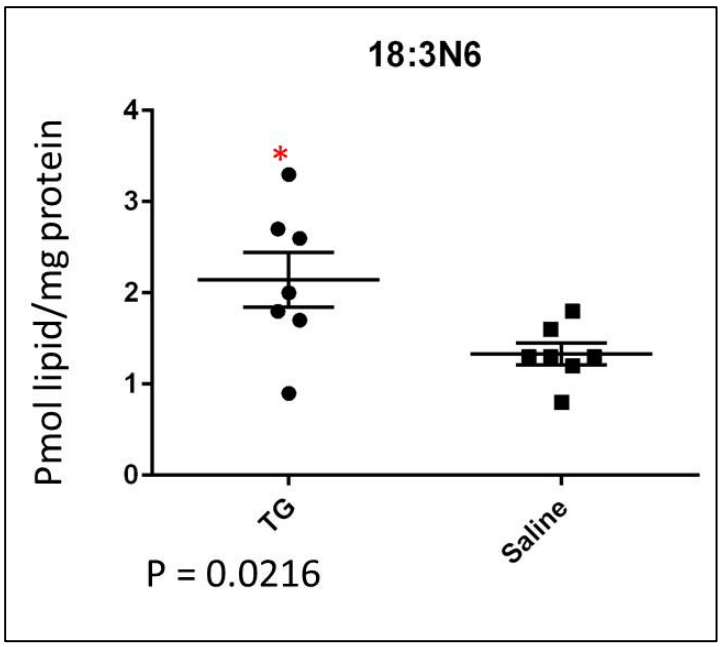
Supplementary Figure 2. Negative control regions for the Striatal probes used. We found no expression of genes outside their known area of expression, indicating that the signals we observed were real. LPL was lightly expressed in these areas, and was shown to on the Allen brain atlas as well (Allen Institute for Brain Science, 2015), however it is important to note that what little signal is observed was actively searched for on the microscope.



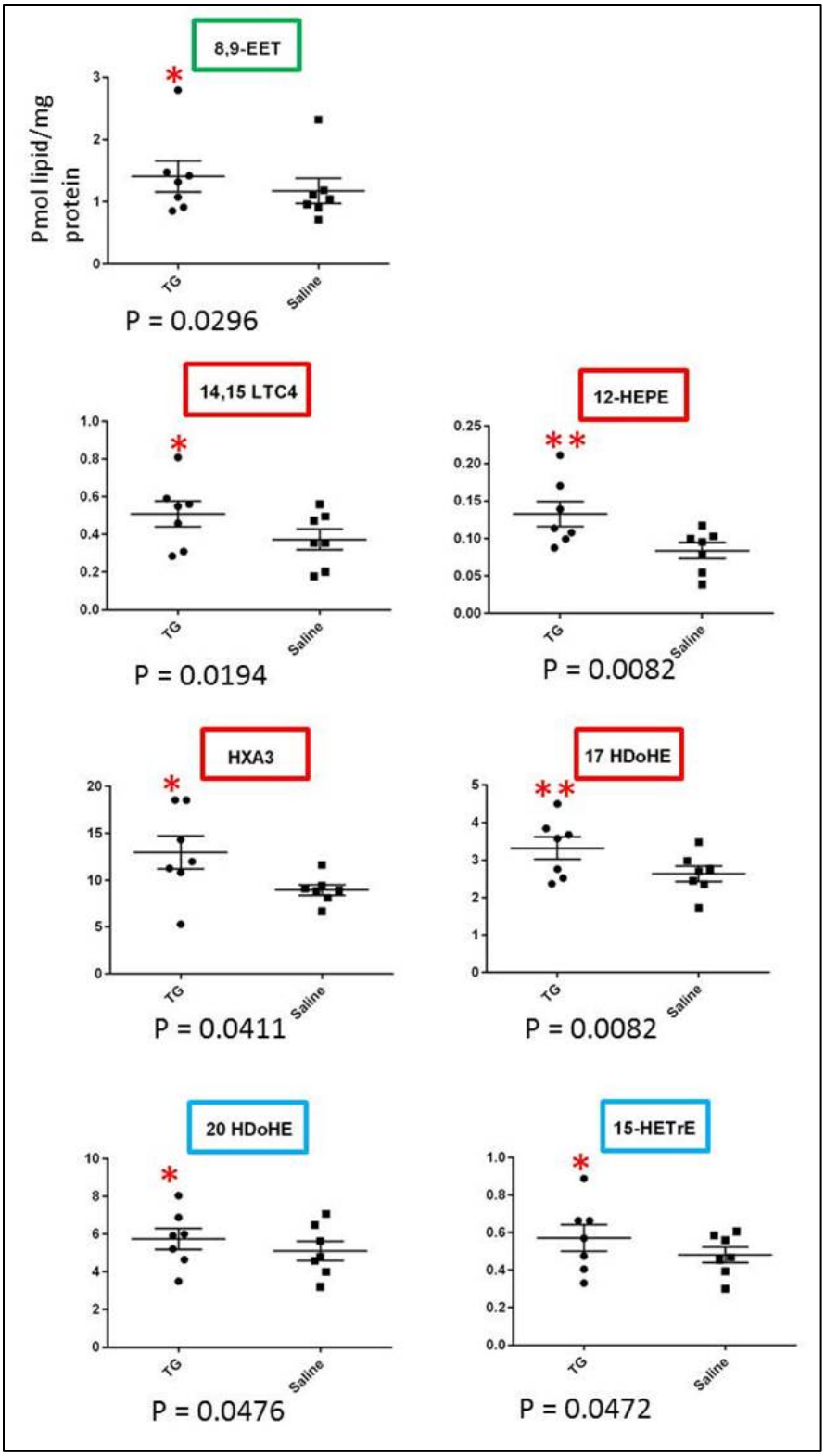
Supplementary Figure 3A. Significant Glycerolipid Changes following BTGD.



Supplementary Figure 3A. Significant Glycerolipid Changes following BTGD, continued.



Supplementary Figure 3B. Significant Free-fatty acid Changes following BTGD.



Supplementary Figure 3C. Significant eicosanoid Changes following BTGD. Items boxed in red indicate LOX pathway byproducts. Green indicates anti-inflammatory compounds and blue compounds indicate derivatives of the elevated FFA species.

Supplementary Table 1A. All Triglycerides assayed.

A. Triglyceride	TG (relative intensity/mg protein)	Saline (relative intensity/mg protein)	P value
54:4 TAG	0.1007	0.06899	**0.0141**
60:9 TAG	0.008994	0.005516	**0.0189**
52:4 TAG	0.08414	0.0579	**0.0202**
54:5 TAG	0.07751	0.05881	**0.0287**
50:2 TAG	0.1594	0.1099	**0.0332**
56:6 TAG	0.2352	0.162	**0.0379**
50:0 TAG	0.4922	0.3527	**0.04**
56:4 TAG	0.03254	0.0229	**0.0459**
52:3 TAG	0.1581	0.1152	**0.048**
60:12 TAG	0.08068	0.05009	**0.0481**
50:3 TAG	0.01944	0.01548	**0.0494**
56:5 TAG	0.06834	0.05221	**0.0533**
52:2 TAG	0.4823	0.3677	**0.0536**
Total TAG	4.655	3.528	**0.0649**
54:1 TAG	0.08915	0.07028	**0.068**
50:1 TAG	0.6237	0.4231	**0.0843**
60:11 TAG	0.02401	0.01862	**0.092**
58:6 TAG	0.01989	0.01411	**0.0938**
48:1 TAG	0.07601	0.06114	**0.0998**
56:1 TAG	0.01691	0.01237	0.1237
56:2 TAG	0.02468	0.01843	0.1397
52:5 TAG	0.0116	0.008278	0.1514
54:6 TAG	0.2151	0.1687	0.157
48:0 TAG	0.302	0.2458	0.1594
60:8 TAG	0.003907	0.003066	0.1807
54:0 TAG	0.09275	0.07746	0.1829
54:3 TAG	0.1694	0.1267	0.2085
60:7 TAG	0.002707	0.001779	0.2224
60:10 TAG	0.03028	0.02441	0.2372
46:1 TAG	0.002947	0.004013	0.2427
52:0 TAG	0.289	0.2338	0.2443
52:1 TAG	0.4078	0.3567	0.2573
50:4 TAG	0.003219	0.002629	0.3412
56:0 TAG	0.009341	0.00744	0.3416
54:2 TAG	0.1765	0.1533	0.4182
48:2 TAG	0.01217	0.01147	0.5624
56:3 TAG	0.02049	0.01917	0.6551

Supplementary Table 1B. All Diglycerides assayed.

B. Diglyceride	TG (relative intensity/mg protein)	Saline (relative intensity/mg protein)	P value
38:5 DAG	0.2913	0.2182	**0.0332**
36:1 DAG	1.411	1.103	0.1119
42:2 DAG	0.03434	0.04288	0.1224
40:7 DAG	0.07781	0.05895	0.1285
38:4 DAG	2.036	1.72	0.141
38:6 DAG	0.2087	0.1508	0.1493
40:1 DAG	0.05983	0.07514	0.1546
32:0 DAG	0.1603	0.1176	0.1595
Total DAG	7.756	6.692	0.193
38:3 DAG	0.06922	0.05607	0.1956
42:9 DAG	0.004439	0.009379	0.2236
46:0 DAG	0.03216	0.02604	0.2278
34:1 DAG	0.862	0.6847	0.2557
32:1 DAG	0.007986	0.01075	0.3331
34:0 DAG	0.3793	0.3324	0.4119
36:3 DAG	0.08296	0.1057	0.4627
40:4 DAG	0.05644	0.049	0.523
38:1 DAG	0.1677	0.1506	0.5242
40:2 DAG	0.03032	0.03717	0.5412
36:2 DAG	0.5956	0.5501	0.5784
36:4 DAG	0.2641	0.2834	0.6694
40:5 DAG	0.03691	0.03446	0.6778
34:2 DAG	0.079	0.07375	0.7253
36:0 DAG	0.3774	0.3608	0.7349
42:8 DAG	0.02382	0.02262	0.7743
40:6 DAG	0.2387	0.2487	0.8038
36:5 DAG	0.01352	0.01255	0.8229
42:1 DAG	0.07688	0.07557	0.9067
38:2 DAG	0.08363	0.08089	0.9174
38:0 DAG	0.02721	0.02675	0.9347

Supplementary Table 1C. All Free-fatty acids assayed.

C. Free Fatty Acid	TG (relative intensity/mg protein)	Saline (relative intensity/mg protein)	P value
18:3 N6	2.143	1.329	**0.0216**
20:3 N9	1.629	1.071	**0.0554**
12:0	79.03	60.7	0.1408
22:5N6	14.73	12.56	0.1527
22:5N3	35.54	30.26	0.1647
20:3 N6	88.37	72.89	0.1649
17:0	81.54	71.36	0.1742
23:0	49.54	34.69	0.2041
22:2	2.5	1.971	0.2256
22:0	69.91	53.91	0.2316
18:0	5348	4466	0.2325
24:1	44.29	30.7	0.2329
20:0	58.06	42.89	0.248
22:1	32.43	25.17	0.2493
20:3 N3	16.67	13.99	0.2549
22:4	155.2	132	0.2739
16:0	4270	3695	0.2741
20:1	169.1	121.3	0.2764
Total	16353	14007	0.2775
20:5	11.03	9.486	0.2779
16:1	131.4	110.9	0.2929
22:6	585.7	513.2	0.3109
18:2	270.3	238.8	0.3541
20:4	2848	2529	0.3563
17:1	5.629	4.486	0.3799
18:1	1675	1455	0.3903
20:2	29.87	25.89	0.4162
24:0	149.7	128.7	0.5435
14:0	42.19	37.66	0.6642
15:0	22	24.54	0.7722
26:0	62.44	60.14	0.8371
18:3N3	1.514	1.529	0.934
18:4	0	0	-
22:3	0	0	-

Supplementary Table 1D. All Eicosanoids assayed.

D. Eicosanoid	TG (relative intensity/mg protein)	Saline (relative intensity/mg protein)	P value
12-HEPE	0.1326	0.08373	**0.0069**
17 HDoHE	3.32	2.637	**0.0082**
14,15 LTC4	0.5084	0.3729	**0.0194**
8,9-EET	1.408	1.176	**0.0296**
HXA3	12.97	8.964	**0.0411**
15-HETrE	0.571	0.4813	**0.0472**
20 HDoHE	5.741	5.106	**0.0476**
12-HETE	15.93	14.85	**0.0476**
16HDoHE	1.927	1.631	**0.052**
15 oxoEDE	0.1504	0.1028	**0.0624**
10 HDoHE	0.9302	0.7425	**0.0894**
PGE2	5.266	6.05	0.1299
13-oxoODE	2.498	2.043	0.1497
12,13 diHOME	0.5673	0.5042	0.1591
5,15-diHETE	0.4836	0.5882	0.1696
5-oxoETE	28.37	23.63	0.1785
HXB3	15.94	11.93	0.1911
8-HETrE	0.6828	0.5684	0.1966
15d PGJ2	0.3894	0.257	0.2316
9-oxoODE	6.941	5.549	0.2344
LTB4	0.1557	0.1413	0.2359
15k PGE2	1.155	0.9549	0.2429
4 HDoHE	8.708	7.924	0.2511
8 HDoHE	1.802	1.598	0.2749
12-oxoETE	1.94	1.502	0.2874
14,15-EET	1.65	1.485	0.2894
9,20 diHOME	0.711	0.6306	0.3002
5,6-diHETrE	0.5959	0.4775	0.3095
13 HDoHE	1.521	1.363	0.3169
13-HOTrE(y)	0.04783	0.07246	0.3209
PGD1	9.452	8.226	0.3376
PGE1	0.246	0.1624	0.3422
PGB2	7.792	5.188	0.3487
5-HETre	0.1406	0.1108	0.3504
8-iso PGF2a III	1.96	1.683	0.3637
11 HDoHE	1.623	1.547	0.3699
11,12-EET	3.484	3.101	0.4029
19-HETE	1.168	0.6267	0.4086
8-HETE	4.192	3.85	0.4185
TXB1	0.06248	0.07104	0.4239
20cooh AA	0.3798	0.2994	0.4484
PGE3	1.572	1.168	0.4505
12-HHTrE	44.98	49.64	0.4544
19(20) EpDPE	1.991	1.159	0.4559
dihomo PGJ2	70.3	61.4	0.4689

Supplementary Table 1D. All Eicosanoids assayed, continued.

A. Eicosanoid	TG (relative intensity/mg protein)	Saline (relative intensity/mg protein)	P value
TxB2	4.865	4.469	0.4883
16(17) EpDPE	0.9826	0.6639	0.4906
16-HETE	3.763	2.421	0.4936
PGD3	3.626	2.812	0.5109
15d PGD2	2.703	2.463	0.5111
18-HETE	0.5279	0.3475	0.5183
5,6-EET	2.484	2.945	0.5197
6k PGF1a1	0.945	1.035	0.5537
11,12-diHETrE	2.235	2.383	0.5593
17-HETE	0.1776	0.1204	0.567
19,20 DiHDPA	1.064	0.7475	0.5683
18-HEPE	0.2397	0.166	0.5722
15-HETE	27.52	26.21	0.5743
8,9-diHETrE	2.255	2.123	0.5968
9,10 EpOME	0.1127	0.1247	0.5983
12,13 EpOME	0.1696	0.1971	0.6237
9-HOTrE	0.06272	0.06952	0.6393
14 HDoHE	2.54	2.361	0.6488
11-HETE	8.521	7.876	0.6496
11-HEPE	0.09516	0.102	0.651
PGJ2	1.713	1.602	0.6703
15-oxoETE	23.11	20.79	0.6766
PGF2a	11.7	10.87	0.7126
9-HEPE	0.1556	0.1724	0.7167
14,15-diHETrE	2.234	2.382	0.719
PGD2	31.17	30.28	0.7206
PGA2	1.32	1.206	0.7375
9-HODE	4.33	4.246	0.7482
8,15-diHETE	1.48	1.441	0.7748
13-HODE	2.594	2.534	0.8012
15k PGF2a	1.54	1.483	0.8226
7,17 dHDPA	0.6292	0.6058	0.8358
dihomo PGF2a	0.2508	0.2546	0.8376
dhk PGD2	8.595	8.38	0.8469
5-HETE	26.87	26.43	0.8582
dhk PGE2	4.325	4.47	0.8634
9-HETE	4.997	4.936	0.8835
LTC4	0.07971	0.07806	0.885
7 HDoHE	0.6379	0.6482	0.8916
5-iso PGF2a VI	2.717	2.745	0.933
dihomo 15d PGD2	56.82	57.65	0.9431
14(15) EpETE	0.02399	0.02343	0.9546
8-iso 15k PGF2b	1.245	1.257	0.9665

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