Dysregulation of Hypothalamic Gene Expression and the Oxytocinergic System by Soybean Oil Diets in Male Mice

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Declarations

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Care and treatment of animals was in accordance with guidelines from and approved by the University of California, Riverside Institutional Animal Care and Use Committee (AUP #20140014 and #20140017).

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Authors' Contributions
Study was designed by PD, MCC, FMS, TJ. Experiments performed by PD, EK, MV, CH, GG, HR, JRE, ET, AR, JR, JV, MCC. Data analyzed by PD, EY, EK, MV, JRD, JML, TJ, FMS and MCC. PD and MCC were major contributors to writing the manuscript and FMS for extensive edits. All authors reviewed and approved the final manuscript.

Abstract

Soybean oil consumption has increased greatly in the past half-century and is linked to obesity and diabetes. To test the hypothesis that soybean oil diet alters hypothalamic gene expression in conjunction with metabolic phenotype, we performed RNA-seq analysis using male mice fed isocaloric, high-fat diets based on conventional soybean oil (high in linoleic acid, LA), a genetically modified, low-LA soybean oil (Plenish) and coconut oil (high in saturated fat, containing no LA). The two soybean oil diets had similar, albeit non-identical, effects on the hypothalamic transcriptome, whereas the coconut oil diet had a negligible effect compared to a low-fat control diet. Dysregulated genes were associated with inflammation, neuroendocrine, neurochemical, and insulin signaling. *Oxt* was the only gene with metabolic, inflammation and neurological relevance upregulated by both soybean oil diets compared to both control diets. Oxytocin immunoreactivity in the supraoptic and paraventricular nuclei of the hypothalamus was reduced while plasma oxytocin and hypothalamic *Oxt* were increased. These central and peripheral effects of soybean oil diets were correlated with glucose intolerance but not body weight. Alterations in hypothalamic *Oxt* and plasma oxytocin were not observed in coconut oil diet enriched in stigmasterol, a phytosterol found in soybean oil. We postulate that neither stigmasterol nor LA is responsible for effects of soybean oil diets on oxytocin and that *Oxt* mRNA levels could be associated with the diabetic state. Given its ubiquitous presence in the American diet, the observed effects of soybean oil on hypothalamic gene expression could have important public health ramifications.

Short Title

Effects of high fat diets on hypothalamic genes

Keywords

Oxytocin, Coconut Oil, Plenish, Diabetes, Linoleic Acid, Stigmasterol

Introduction

The hypothalamus is part of a complex network in the brain that is responsible for sensing nutritional status and executing behavioral and metabolic responses to changes in fuel availability. As part of this network the hypothalamus produces intrinsic peptides and neurotransmitters that influence food intake energy balance and glucose homeostasis (1,2). One such hypothalamic anorexigenic peptide is oxytocin (OXT), which is synthesized in the magnocellular neuroendocrine cells (MNCs) and acts as a peripheral hormone after being released from axons in the posterior pituitary into the systemic circulation. Oxytocin is also released centrally from the soma and dendrites of MNCs within the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus [(3,4)]. Another component of the central oxytocinergic signaling is the oxytocin-synthesizing parvocellular neurons of the PVN that project to autonomic brainstem regions and likely mediate leptin anorectic signals (2). Expression of *Oxt* and the related neuropeptide, vasopressin (*Avp*), is increased in both induced and spontaneous models of diabetes (5). Centrally, oxytocin is involved in feeding regulation and energy expenditure. For example, mice lacking OXT or its receptor exhibit mild or late-onset obesity, although normal body weight has also been reported (6).

It has been shown that energy-dense, high-fat diets (HFD) disrupt energy balance and cause changes in body weight regulation and an upregulation of proinflammatory cytokines and insulin resistance in the hypothalamus and other tissues (7–9). While it is clear that the hypothalamus is a key player in controlling the balance

between energy homeostasis and obesity/diabetes, the specific mechanisms regulating that balance remain elusive. Even less well-studied is the impact of specific components of the diet on the hypothalamus.

Individual risk factors such as the consumption of fat-rich foods and a sedentary lifestyle are thought to contribute significantly to the rapid rise in the prevalence of obesity and comorbid diseases such as diabetes (10,11) . While the vast majority of diet-induced obesity studies focus on the role of saturated fats, such as those found in animal fat, a growing body of evidence suggests that polyunsaturated fatty acids (PUFA), such as those found in vegetable oils, also contribute to the obesity epidemic (12–17). For example, there has been a 1000-fold increase in the consumption of soybean oil in the U.S. during the 20th century and, as a result, the *per capita* consumption of its primary unsaturated fatty acid component, linoleic acid (LA, C18:2), has increased from <1 to ~7.4% of energy intake (18). Soybean and other oils high in LA have been shown to be obesogenic and diabetogenic in rodent systems by us and others (12–14,16) and we have shown that a diet enriched in soybean oil similar to the American diet causes a global dysregulation of hundreds of genes in the liver compared to an isocaloric coconut oil diet (14).

While neither our study, nor any other we could find in the literature, has examined the impact of a soybean oil diet on the hypothalamic transcriptome, alterations in dietary PUFAs such as LA and omega-3 fatty acids and their metabolites have been shown to modulate fatty acid levels in different regions of the brain, including the hypothalamus (19,20). Unsaturated fats have also been shown to impact food

intake, glucose homeostasis and gene expression in the brain (21–24). In particular, LA has been proposed to target specific orexigenic (*Agrp*) and anorexigenic (POMC, Cart) neuronal populations in the hypothalamus (16) and to increase levels of hypothalamic arachidonic acid (AA), a potent proinflammatory molecule (25). Thus, the obesogenic and diabetogenic properties of a soybean oil-based diet may be due to modulation of pro-inflammatory processes and/or hypothalamic signaling of key neuropeptide populations by LA.

Non-fatty acid components in soybean oil include phytosterols such as stigmasterol, campesterol and β-sitosterol. Of these, stigmasterol has been shown to have metabolic effects such as increasing insulin synthesis and secretion and causing cholesterol efflux in macrophage foam cells (26,27). The metabolic effects of stigmasterol may be due to its role as a ligand for two nuclear receptors: it is an agonist for the liver X receptor (LXR, *Nr1h3*) [28,29], which is expressed in the hypothalamic nuclei, and an antagonist for the farnesoid X receptor (FXR, *Nr1h4*) (30). Since both nuclear receptors are linked to food intake, energy expenditure and glucose and lipid homeostasis (31,32) , stigmasterol may influence the development of obesity and/or diabetes by a diet enriched in soybean oil.

In this study, we hypothesized that a soybean oil-rich diet, such as that currently consumed in the U.S., impacts hypothalamic gene expression and oxytocin peptide levels differentially from other HFDs, that those changes correlate with obesity and/or diabetes and that they involve one or both of its major components, LA and stigmasterol. To test these hypotheses, we subjected male C57BL/6N mice to four isocaloric HFDs comprised of coconut oil (largely medium chain saturated fats),

conventional soybean oil (high in LA), a genetically modified soybean oil low in LA (Plenish) or coconut oil supplemented with stigmasterol. We performed RNA-seq analysis on the hypothalamus of the mice fed the first three diets (and a low-fat control diet) and analyzed hypothalamic and peripheral levels of oxytocin in mice fed all four diets. The results show that compared to the coconut oil and low-fat control diets, the two soybean oil-based diets resulted in a significant dysregulation of more than 100 hypothalamic genes including those involved in neurochemical and neuroendocrine pathways and metabolic and neurological disorders. With only two exceptions, the changes in gene expression were not significantly different between the high-LA and low-LA soybean oil diets. Importantly, oxytocin was significantly upregulated on both the RNA level in the hypothalamus and the peptide level in the plasma in both soybean oil diets, but not the coconut oil diet. In contrast, immunohistochemical (IHC) staining of the SON and PVN in the hypothalamus showed reduced OXT protein levels in both sets of soybean oil-fed mice, consistent with increased peripheral release of OXT hormone. The stigmasterol diet did not impact hypothalamic oxytocin expression or plasma levels nor promote obesity or diabetes compared to the coconut oil diet. Taken together, our results support the hypothesis that a soybean oil-enriched diet impacts gene expression in the hypothalamus, including *Oxt,* and causes an elevated level of circulating oxytocin. While these effects correlated significantly with diabetes as measured by a glucose tolerance test (GTT), they did not correlate with body weight nor seem to involve either LA or stigmasterol.

Methods

Diets

Three isocaloric diets with 40 kcal% fat (4.87 kcal/gm) (Table 1) were formulated in conjunction with Research Diets, Inc. (New Brunswick, NJ). The diets are based on the Surwit diet, which is widely used in diet-induced obesity studies and formulated with elements from the AIN-93 diet. The 5% fiber from cellulose in the AIN diet is replaced with cornstarch. These 40 kcal% diets have been described previously (13,14) and include: 1) coconut oil (CO) providing 36 kcal% from CO and 4 kcal% from conventional soybean oil (SO) to provide the essential fatty acids LA and ALA; 2) SO+CO providing 21 kcal% fat calories from CO and 19 kcal% from SO, of which 10 kcal% are from LA; 3) PL+CO in which conventional SO was replaced on a per gram basis with a genetically modified high oleic oil, Plenish (PL;DuPont Pioneer, Johnston, IA). A fourth diet (ST+CO) contained 40 kcal% fat from CO plus 0.1gm stigmasterol (Sigma-Aldrich S2424), equivalent to the amount that a 40 kcal% SO diet would contain. To determine the contribution of LA to our observed phenotype, we chose a control fat that is plant based but high in saturated fat and low in LA, i.e. coconut oil. Furthermore, to make the diets isocaloric, without increasing the LA content in SO+CO, we chose to add some coconut oil to our other two HFDs. This design allowed us to test the effect of saturated fat without LA (CO) versus unsaturated fats with different levels of LA. SO+CO had higher levels of LA than PL+CO thus allowing us to elucidate the effect of LA. CO versus PL+CO allowed investigation of the effect of saturated versus unsaturated fats. The fatty acid (Table 2) and phytosterol (Table 3) composition of Plenish, conventional SO and CO used in the diets were determined by Covance Laboratories (Madison, WI). The total amount of carbohydrates and protein were constant across all diets, including

the low fat control: vivarium chow (Viv) (Purina Test Diet 5001, Newco Distributors, Rancho Cucamonga, CA) with 3.36 kcal/gm fat. Viv chow also contains ~25% fiber.

Diets were provided to the animals in pellet form, twice weekly for up to 24 weeks; the amount of food consumed was calculated weekly on a per cage basis. Food intake (in terms of total calories consumed) was not significantly different between any of the HFD groups. The Viv chow group consumed a greater quantity of food by mass because it has a much lower caloric density.

Animals

Care and treatment of animals was in accordance with guidelines from and approved by the University of California, Riverside Institutional Animal Care and Use Committee (AUP #20140014 and #20140017). All mice had *ad libitum* access to food and water (other than the indicated fasting times). At the end of the study, some mice were sacrificed by CO_2 inhalation and their brains snap-frozen. Others were subjected to tail blood draw or cardiac puncture and sacrificed by cervical dislocation, or transcardial perfusion under isoflurane anesthesia (see below), in accordance with stated NIH guidelines.

Male C57BL/6N mice (Charles River Laboratories) were bred in-house and maintained on a 12:12 h light-dark cycle in a specific pathogen-free vivarium (SPF) for the 40 kcal% diet experiment (Cohort 1) (Fig. 1A). Pups were weaned at three weeks of age and started on one of the four different diets (Viv Chow, CO, SO+CO, PL+CO). At least 12 mice were placed on each diet with three to four animals housed per cage.

Mice were sacrificed (by CO_2 inhalation) at 24 weeks after weaning and their brains harvested and stored at -80 ^{o}C for subsequent transcriptomic analysis. To isolate the

hypothalamus brains were slightly thawed in a dry-ice filled container and blocked manually at the anterior commissure rostrally, at mammillary bodies caudally (1 mm rostral to end of median eminence), and at the top of third ventricle dorsally and then the entire hypothalamus was removed. Glucose tolerance, food consumption and body weight data from these mice have been published previously (13).

Cohort 2 mice, housed in a conventional vivarium, were subjected to Viv chow, CO, SO+CO, PL+CO or ST+CO diets starting at 3 weeks of age and their tissues were used in immunoassay, immunohistochemical and qPCR experiments (Fig. 1A). Weekly change in body mass (expressed as body weight) per mouse was measured in grams for up to 16 weeks after the start of diets. Food consumed on a given diet was measured on a per cage basis, normalized to the number of mice per cage. Food was changed and measured twice weekly; values were combined to generate the weekly average. Glycemia after fasting and glucose load (glucose tolerance, GTT) were also measured, as described previously (13,14), after 16 weeks on the diets. Mice were sacrificed one to several weeks later. Some mice were subjected to cardiac puncture under isoflurane anesthesia for collection of blood for use in immunoassay experiments. Other mice were transcardially perfused for use in brain immunohistochemical experiments. For a subset of mice, sacrifice consisted of CO_2 inhalation followed by decapitation in order for brains to be snap frozen for use in qPCR experiments.

Glucose Tolerance Test

GTT was performed as described previously (14). Briefly, mice were fasted overnight and then injected with D-glucose 2g/kg body weight by an intraperitoneal injection of a sterile 20% glucose solution in 0.9% saline. Under light restraint tail blood was drawn and glycemia measured before (0 min) and after injection (at 15, 30, 60 and 120 min). Area under the curve (AUC) was plotted for group comparisons.

RNA Extraction and Sequencing

Total RNA was isolated from hypothalamic homogenates using a miRNeasy kit (Qiagen, Inc., Valencia, CA) and evaluated for purity and concentration by NanoDrop (NanoDrop Technologies,Wilmington, DE) and for quality using Agilent 2100 Bioanalyzer (Santa Clara, CA). Poly(A)$^+$ RNA (4 µg) having an RNA Integrity Number (RIN) of >7.5 was used to construct sequencing libraries with the TruSeq Long RNA Sample Prep Kit (Illumina; San Diego, CA). RNA libraries were validated for RNA integrity by Bioanalyzer (Santa Clara, CA), pooled in equimolar amounts, and sequenced on an Illumina HiSeq 2000 at the UCR Genomics Core to generate 50 base, paired-end reads. Three biological replicates each for Viv Chow, CO, SO+CO and PL+CO were sequenced yielding ~15 million reads per sample.

Differential gene expression analysis using RNA-seq data

Reads were aligned to the mouse reference genome (mm10) with TopHat v2.1.1 using the default parameters with the exception that only one unique alignment for a given read was allowed. Raw read counts were calculated at the gene level for each sample using HTSeq v0.6.1. Library normalization was performed with EDASeq [33]; within-lane normalization on GC content was performed with the LOESS method and

between-lane normalization was performed with a non-linear full quantile method. Normalization factors from EDASeq were used for differential expression analysis with DESeq2. Normalized read counts, and FPKM (fragments per kilobase per million), and rlog (regularized log transformation) results were generated for downstream analysis. Heatmaps were generated with pheatmap package in R using rlog values. Genes that had a log2 fold change $\geq$.5 and padj $\leq$ .5 were included in the heatmap; data were row-normalized before plotting.

The gene expression patterns of the hypothalamic samples from two biological conditions can be characterized by a high dimensional vector that consists of the read counts of all differentially expressed genes. To visualize the similarity in the gene expression patterns, we conducted a principal component analysis (PCA) to transform the high dimensional vectors into ones in a three-dimensional space. In the PCA, the read counts in a vector were normalized by the size of the corresponding RNA-seq library. The three dimensions of the transformed space were chosen by the three principal components with the highest Eigenvalues. The Euclidean distance in the three dimensional space was used to measure the similarity of the gene expression patterns between the corresponding treatment group samples.

Hypothalamic genes that were significantly (*P* and *Padj* or q $\leq$ .05, at least 1.5 fold-change) dysregulated between any two dietary comparisons were uploaded to Panther (http://pantherdb.org) (34) for functional annotation clustering (35). Lists of various disease-associated mouse genes were generated using Pubmed and cross-referenced with genes significantly altered between the diets as described before (14). Venn

diagrams were created using the free online tool VENNY (36). The processed RNA-seq data is available online (37).

Quantitative Polymerase Chain Reaction (qPCR)

Following 18-26 weeks of chronic diet and GTT, mice were sacrificed by decapitation. Whole brains were collected, snap-frozen in 2-methylbutane over dry ice and stored at -80ºC until later use. From each brain, the hypothalamus was dissected in half and homogenized in TRIzol Reagent (Thermo Fisher Scientific). Total hypothalamic RNA was prepared using a modified phenol-chloroform extraction protocol with the Qiagen RNeasy Plus Mini Kit. RNA concentration and purity were evaluated using a spectrophotometer (Nanodrop; Thermo Scientific). Reverse transcription qPCR was performed with the SensiFAST SYBR No Rox One-Step Kit (Bioline) on the CFX Connect Real-Time PCR Detection System (Bio-Rad) using the following 2-step cycling protocol: 45°C for 10 min, 95°C for 2 min, followed by 40 cycles at 95°C for 5 sec, 20 sec at primer's ideal annealing temperature and melt curve analysis at 65°C and 95°C for 5 sec. Each reaction was run in triplicate using a total of 10 ng of RNA per reaction well.

Oligonucleotide primer sequences (Table 4) were custom-designed and synthesized by Integrated DNA Technologies or ordered as pre-designed assays. Primers were designed to meet several criteria using NCBI Primer Blast. For selectivity, primers were optimized by testing against cDNA generated from whole mouse hypothalamus using RT-qPCR and gel electrophoresis. Only primers that gave single-band amplicons in the presence of RT and that matched the base length of the

predicted target were chosen for further annealing temperature optimization and efficiency checks via qPCR. A temperature gradient from 54-62°C was used to determine optimal annealing temperature for each primer. Efficiency curves were generated over a 4 to 6 point template concentration range (100 ng–1 pg). Selected primers were optimized to yield 90-110% efficiency. No-template controls were run to rule out extraneous nucleic acid contamination and primer dimer formation. Negative reverse transcriptase controls were used to rule out any potential genomic DNA contamination present in the RNA preparation.

Samples were excluded from analysis if they had low amplitude peaks, two or more amplicons generated on melt curves, individual Cq values greater than 30 or having more than 1.0 standard deviation from mean Cq of technical replicates. The fold-change gene expression of *Oxt* relative to *Actb* (beta-actin) was determined using the Pffalf method and biological replicates from CO as the control group [38]. A total of 18 hypothalamus RNA samples from 18 mice were analyzed.

Immunohistochemistry

At sacrifice mice were anesthetized with isoflurane and subjected to cardiac puncture for collection of blood. They were immediately perfused transcardially with ice-cold 0.01 M phosphate-buffer (PB) plus 0.9 g% NaCl (PBS) followed by 4% paraformaldehyde (PFA) in PB (pH 7.4). Brains were dissected, sucrose-infiltrated (30% sucrose) and once cryoprotected were covered with optimal cutting medium (Tissue-Tek), cryosectioned at 40-mm thickness, mounted on slides and stored at -20ºC until use. Brains were removed *ex vivo* (see above), and post-fixed in PFA for 24 hours at 4ºC and subsequently cryoprotected in 30% sucrose until brains were submerged

(about 24 hours). Brains were cryosectioned into 30-µm sections and mounted onto gelatin-subbed microscope slides. Sections were washed free of PFA and subjected to a permeabilization/blocking step using 4% normal donkey serum, 1% BSA, 0.3% Triton-X in PBS. Sections were then incubated with one of two primary antibodies: mouse monoclonal anti-oxytocin neurophysin (PS38, 1:100; gift of Dr. H. Gainer, NIH) (39) for 48 hours at 4ºC. This antibody has been shown to reliably detect levels of OXT (40). Sections were then washed in PBS three times and incubated with a secondary antibody (donkey anti-mouse Alexa 594, 1:1000) (41) at RT for 1.5 hours. After additional washes at 4ºC, slides were cover-slipped using vectashield containing DAPI (Vector Labs). OXT immunoreactivity of hypothalamic sections was analyzed under an Axiophot fluorescence microscope (Zeiss) and images acquired as fluorescent micrographs using a SPOT-II digital camera (Diagnostic Instruments). A mouse brain atlas [42] was referenced to ensure neuroanatomical comparison of equivalent brain regions. A total of seven experiments were performed using three sections from SON and PVN in each of 28 mice (n=4-6/group).

Oxytocin Immunoassay

Blood was collected by cardiac puncture and the plasma was separated at 2,000xg for 20 min using a refrigerated centrifuge at 4ºC. Samples were subjected to solid-phase extraction using 200 mg C18 Sep-Pak columns (Waters) using a modification of a published protocol (43). Columns were equilibrated with 3 mL of acetonitrile, then washed twice with 3 mL of 0.1% trifluoroacetic acid (TFA). Plasma (100 µL) was mixed with 4X volume of 0.1% TFA, centrifuged at 17,000 g for 15 min at 4ºC, and the acidified and clarified plasma was then applied to the column. The

columns were washed once with 15 mL of 0.1% TFA; the flow-through fraction was discarded. Analyte was collected by elution with 3 mL of 60% acetonitrile and completely dried under vacuum evaporation. Plasma samples were subjected to an enzyme-linked immunosorbent assay (EIA) as indicated by manufacturer (Enzo Life Sciences) (44).

The assay is highly specific for OXT and does not detect vasopressin. Cross-reactivity of the kit is reported as mesotocin (7%), OXT (100%), Arg^8-Vasotocin (7.5%), and <0.02% for other related molecules. Specifications of this kit are 15 pg/ml (or 1.5 pg/well) for limits of detection and 9% and 7% for intra- and inter-assay variability, respectively. The lyophilized sample was reconstituted in 0.12 mL of assay buffer provided in the kit and diluted 1:4 prior to processing. Briefly, standards and samples (100 µl) were loaded in duplicate onto a 96-well ELISA plate and processed as directed by kit specifications. Optical density (OD) was read at 405 nm; absorbance values were manually corrected using average OD values obtained from the control “blank” wells. OXT concentration values were determined according to a four-parameter logistic curve standard curve. Values were reported as pg/mL. Extraction efficiency (recovery >80%) was determined by spiking plasma with the 200 pg/ml oxytocin standard and comparing to unspiked plasma. Values obtained for control mouse subjects were similar to those reported previously (45). A total of 29 samples were evaluated for plasma OXT.

Nongenetic statistical analysis

Data are presented as mean +/- standard error of the mean (SEM). Hypothesis testing was conducted using statistical analysis (GraphPad Prism 6.0) using the following tests: repeated measures two-way ANOVA for effects of diet and time on body

weight gain and food consumption and one-way ANOVA for effects of diet on GTT and EIA and qPCR. ANOVAs were followed by Tukey's, Fisher's LSD and Bonferroni post-hoc tests for multiple comparisons. For simple planned comparisons we used one-tailed unpaired Student's t-test. Statistical significance was accepted at an alpha level less than or equal to .05. Linear regression analysis was performed between body weight, glucose tolerance and mRNA and plasma levels of oxytocin. The following cut-offs were used to determine significance: Pearson's coefficient $r > 0.5$ with $P \leq 0.05$ and $R^2 > 0.5$.

Sample sizes were calculated using power analysis at a desired statistical power of .8 and alpha level of .05 (www.stat.ubc.ca and masc.org.au\stats\PowerCalculator).

RESULTS

Dietary oils have differential effects on hypothalamic gene expression

Transcriptomic analysis (RNA-seq) was performed on hypothalami obtained from adult male C57BL6/N mice used in a previous study (Cohort 1) in which we reported that a HFD made with conventional soybean oil (SO+CO) induces more obesity than isocaloric diets made with either coconut oil (CO) or a genetically modified low-LA soybean oil, Plenish (PL+CO) (13) (Fig. 1A). As described previously (14) , these diets were designed to have a level of total fat (40% kcal) and LA (10% kcal) comparable to the current American diet. Coconut oil was used as the HFD control as it is comprised nearly exclusively of saturated fats and contains no endogenous LA; a small amount of soybean oil had to be added as LA is an essential fatty acid (see Tables 1 and 2).

Principal component analysis (PCA) showed that both soybean oil diets significantly alter gene expression in the hypothalamus compared to the CO diet, which

was very similar to the low-fat vivarium control diet (Viv chow) (Fig. 1B). In fact, a heat map of differentially expressed genes (DEGs) significantly (log2 fold-change $\geq$.5 and padj $\leq$ .5) dysregulated in at least one of the three HFDs versus Viv chow revealed a nearly identical transcriptome in the CO diet and Viv chow: only five genes were significantly different (*Col11a1, Rgs16, Gm43398, Atp2a1, Ppp1r3a*) (Supplementary Table 1) (46). In addition, the two soybean oil diets (SO+CO and PL+CO) have a similar pattern of gene expression which is roughly the inverse of that observed in Viv chow and CO-fed mice (Fig. 2A). In fact, expression of only one gene was significantly different between SO+CO and PL+CO: *Kcng1* (Fig. 2A, asterisk and Fig. 2C) as well as a putative gene *1700018M17Rik* (not shown). *Kcng1* is a potassium voltage-gated channel modifier (subfamily G member 1) which belongs to a family of proteins that regulate neurotransmitter release, among other physiological functions: its expression was downregulated ~2-fold in the conventional soybean oil compared to the Viv chow but not changed by either coconut or Plenish oils.

The top half of the heatmap contains the genes that have a higher level of expression in Viv chow-fed mice compared to at least one of the HFD groups ("Up in Viv Chow") while the bottom half contains the genes that have lower levels of expression in Viv chow-fed hypothalami ("Down in Viv Chow"). Gene ontology (GO) analysis of the molecular functions associated with the dysregulated genes revealed that the majority of the genes in "Up in Viv Chow" (down in SO+CO and PL+CO) are categorized as "binding" and "catalytic activity" genes (Fig. 2B top). The binding category contains genes that code for nucleic acid or DNA binding proteins such as transcription factors *Etv1* and *Neurod6* and RNA adenosine deaminase *Adarb1* (Fig. 2A, solid black arrows).

The proto-oncogene *Wnt3*, which signals to the transcription factor β-catenin and the neuropeptide genes *Oxt* and *Avp* (Fig. 2A, pink arrows), are also included in the binding category. The catalytic activity genes suppressed by the soybean oil diets ("Up in Viv Chow") include G-protein modulators (*Rgs4* and *Rgs16)*, a voltage-gated potassium channel (*Kcnab2)* and a mitochondria reductase (*Msra)* (Fig. 2A, red arrows). The genes "Down in Viv Chow" (up in SO+CO and PL+CO) were also primarily in "binding" and "catalytic activity" categories (Fig. 2b bottom). The binding category includes *Rps13, Six6* and *Cngb1* (Fig. 2A, green arrows) while the catalytic activity category includes protease inhibitors (*Itih3, Pcsk1n)*, glucokinase (*Gck)* and a peroxidase (*Gpx3)* (Fig. 2A, blue arrows and Fig. 2C). (See Supplementary Table 2 for genes included in each GO category (46)).

When compared to the Viv chow-fed mice there were 228 hypothalamic genes that were significantly dysregulated in SO+CO and 138 genes in PL+CO-fed mice (Fig. 3A and Supplementary Tables 3, 4 (46)). There was a smaller number of genes dysregulated in the two soybean oil diets when compared to the isocaloric CO diet: 120 genes (87 up- and 33 down-regulated) in SO+CO and 86 genes (82 up- and four down-regulated) in PL+CO (Fig. 3A and Supplementary Tables 5, 6 (46)). *Hist1h2ai,* a gene involved in nucleosome assembly [47], was increased the most (~11-fold) in SO+CO and *Hmx3,* a transcription factor associated with hypothalamic development and neuroendocrine function [48,49], was increased the most in PL+CO (~10-fold) (Fig. 3B top). For both soybean oil diets, the predicted gene *Gm44367* was the most upregulated (18-fold, not shown). *Aspn,* which is involved in regulating inflammatory responses (50), and *Ankk1* were the most down-regulated genes in SO+CO *(*~3-fold) and PL+CO (~21-

fold) versus CO, respectively (Fig. 3B, bottom). (The putative gene *1700018M17Rik* was also highly down-regulated, not shown). Interestingly, *Ankk1* alters dopamine D2 receptor signaling; genetic variants in it have been associated with obesity [(51)], neuropsychiatric disorders, impulse control disorder and exercise reinforcement (52,53).

Hypothalamic genes dysregulated by soybean oil are linked to neuroendocrine, neurochemical, signaling and gene regulation pathways

GO analysis was performed on the genes dysregulated versus CO that were common in both soybean oil diets (51 genes), as well as the genes uniquely dysregulated by SO+CO (69 genes) and PL+CO (35 genes) (Fig. 4A and Supplementary Table 7 (46)). Of the 69 genes dysregulated in SO+CO versus CO, only 31 could be matched to one of 27 biological pathways, including neurochemical and neuroendocrine pathways, inflammatory processes and insulin signaling (Fig. 4A). The neurochemical and neuroendocrine pathways represented here include cholecystokinin signaling (*Hdc)*, thyrotropin-releasing hormone receptor (*Trh),* gonadotropin-releasing hormone receptor *(Gck)* and sex steroid hormone biosynthesis (*Hsd17b7)* (Fig. 4B). Dysregulated genes involved in inflammation include *Col6a1* which signals via chemokines or cytokines and *Cited1, a Cbp/p300 Interacting Transactivator that* signals through transforming growth factor beta (TGF-beta) (Fig.4B). Dysregulated genes involved in insulin signaling include *Irs4* (insulin receptor substrate 4) and *Prkcg* (protein kinase C gamma) (Fig. 4B). In addition to insulin signaling, *Prkcg* is involved in acetylcholinergic, adrenergic, oxytocin receptor-mediated, thyrotropin releasing hormone and Wnt signaling and has a wide distribution and range of function in the

brain (54–56). The expression and/or function in the brain has also been documented for *Hdc (57,58)*,*Trh (59), Cited1 (60,61)Gck (62), Irs4 (63).*

In the PL+CO vs CO comparison two of the 35 dysregulated genes appeared in multiple pathways: *Frs3*, which affects angiogenesis and fibroblast growth factor (FGF) signaling, and *Jund,* which modulates gonadotropin releasing hormone (GnRH) receptor, inflammation and TGF-beta mediated (Fig. 4B). Only two of the 51 commonly dysregulated between SO+CO vs CO and PL+CO vs CO were associated with biological pathways in the GO analysis: vasopressin (*Avp)* and oxytocin (*Oxt)* (both increased ~3-fold compared to CO) (Fig. 4C) are associated with opioid prodynorphin and vasopressin synthesis. Interestingly, hypothalamic expression of *Jund* is up-regulated following physiological activation of vasopressinergic and oxytocinergic MNCs of the SON (64) (Fig. 4B). It is worth noting that all of the soybean oil-dysregulated genes highlighted in Fig. 4 trended in the same direction in the high-LA (SO+CO) and low-LA (PL+CO) diets, even if the expression in one of the diets did not quite reach significance.

Hypothalamic genes dysregulated by dietary fat are linked to several types of molecular functions including transcriptional regulation

GO analysis with respect to molecular function revealed additional genes of interest dysregulated by the soybean oil diets (Supplementary Fig. 1 and Supplementary Table 7) (46). The SO+CO diet resulted in an upregulation of *Gal*, *Gabre, Gpr50*, *Mc3r* and downregulation of *Sema7a* and *Shisa6.* In the PL+CO versus CO comparison only one gene was dysregulated: *Crlf2* (up). The transcriptional regulators that were dysregulated in SO+CO versus CO include *Neurod6* (down), *Etv1*

(down) and *Six6* (up). *Neurod6* is known to play a role in neurogenesis in the hypothalamus (65). Hypothalamic expression of *Etv1* evokes an increase in water intake behavior (66). *Six6* is required for neuroendocrine development, specifically that of the suprachiasmatic nucleus, the expression of which is regulated in a sex-specific and circadian manner (67,68).

Hypothalamic genes dysregulated by soybean oil diet are linked to metabolic diseases and inflammation

To reveal additional links between the soybean oil diets and metabolic disease, we compared Pubmed-generated lists of genes associated with diabetes, lipid metabolism, obesity (grouped as metabolic disease-related) and inflammation to the hypothalamic DEGs. Eighteen disease-associated genes were identified across the four categories for SO+CO versus CO, of which 11 were uniquely dysregulated by SO+CO: *Hsd11b1, Gck, Hdc, Ghsr, Hsd17b7, Gal, Dio2, Mc3r, Gpx3, Nr4a2, Sema7a* (presented in order of decreasing fold-change) (Table 5). In contrast, there were nine disease-associated genes that were identified for PL+CO versus CO with *Mt1* and *Crlf2* being the only genes that were uniquely dysregulated by PL+CO (Table 5). The genes commonly dysregulated in both soybean oil diets were *Hcrt, Oxt, Avp, Cartpt, Mmp11* and *Abhd8* (Table 5). Interestingly, when compared to Viv chow, both SO+CO and PL+CO diets caused dysregulation of eight to 22 genes associated with metabolic disease (Supplementary Table 8) (46) while the CO diet yielded only two disease-related genes (*Atp2a1*, *Rgs16*), both of which are linked to inflammation. This suggests that the medium chain fatty acids in coconut oil not only have less of an impact on

hypothalamic gene expression, in general, but also in terms of genes expression, related to metabolic and inflammatory diseases than the two soybean oil diets (Table 5).

Hypothalamic genes dysregulated by soybean oil diet are linked to neurological diseases

A similar analysis was performed to identify hypothalamic DEGs related to neurological diseases using Alzheimer's disease, anxiety, autism, depression, pain, Parkinson's disease and schizophrenia as search terms in Pubmed. As with inflammation and metabolic diseases, more genes in the SO+CO versus CO comparison were identified in one or more categories than the PL+CO versus CO comparison. No genes associated with neurological diseases were identified among the CO versus Viv chow DEGs (Table 6). A Venn analysis of dysregulated genes in SO+CO and PL+CO versus CO that are associated with metabolic diseases plus inflammation categories (Table 5) and with neurological disorders (Table 6) reveal a total of nine common genes (Fig. 5A). Those genes include: *Hcrt* (metabolic disease, anxiety, depression and pain), *Gal* (metabolic disease, Alzheimer's, anxiety, depression and pain), *Nr4a2* (inflammation, Parkinson's disease and schizophrenia), *Mt1* (metabolic disease, inflammation and depression), *Dio2* (metabolic disease, inflammation, anxiety and depression), *Ghsr* (metabolic disease, inflammation, pain and Parkinson's disease) (Fig. 5B) and *Hdc* (metabolic disease and anxiety), *Avp* (metabolic disease, anxiety and pain), *Oxt* (metabolic disease, inflammation, anxiety, autism, depression and pain) (Fig. 4B,C). Importantly, *Oxt* was the only gene dysregulated in *both* soybean oil diets versus *both* CO and Viv chow and also associated with all three

disease categories: metabolic disease, inflammation and neurological disorders (Fig. 5C).

Metabolic phenotype induced by soybean oil is not due to stigmasterol

Since the RNA-seq analysis of the hypothalamus of mice fed both the conventional, high-LA (SO) and genetically modified, low-LA (PL) soybean oils revealed similar effects on the expression of *Oxt* (and many other genes), we designed a second cohort of mice to determine whether another component of the oils – stigmasterol -- might play a role in the altered *Oxt* expression and obesity and diabetes. Mice in Cohort 2 (Fig. 1A) were fed Viv chow, CO, SO+CO, PL+CO and ST+CO (CO diet supplemented with 0.1 gram stigmasterol per 1.1 kg diet to match the level in the two soybean oil diets, Table 1). Consistent with our previous studies (13,14), we observed that all three HFDs in Cohort 2 mice fed CO, SO+CO or PL+CO experienced a greater gain in body weight than the Viv chow-fed mice after 16 weeks on the diet. A two-way ANOVA showed main effects of duration on diet ($F_{4,179}$=824, p<.0001) and diet ($F_{3,179}$=91 p<.0001). Post-hoc group comparisons indicated that the SO+CO group gained the most weight (36.6 versus 24.3 grams for Viv chow) (Fig. 6A). However, there was no significant difference in body weight in PL+CO mice compared to the CO mice in Cohort 2 after 16 weeks on the diet (31.4 and 31.2 grams, respectively) (Fig. 6A), as we did previously (13). This discrepancy could be due to the fact that mice in Cohort 2 were on the diets for only 16 weeks compared to 24 weeks in Cohort 1: the difference in weight between PL+CO and CO mice in that study did not gain significance until 12 weeks on the diet [13]. Another possible explanation is that the mice in the 24-week study (Cohort 1) were housed in a specific pathogen-free animal facility, whereas mice

for the 16-week study (Cohort 2) were housed in a conventional vivarium, potentially implicating the microbiome. Both soybean oil diets (SO+CO and PL+CO) made the mice more glucose intolerant compared to the CO and Viv chow diets (Fig. 6B), also consistent with our previous results (13).

Interestingly, we found that mice fed the ST+CO diet gained less weight compared to SO+CO mice but the same amount of weight (31.7 grams) as the CO and PL+CO diets. The ST+CO-fed mice also showed no signs of glucose intolerance in the GTT and were trending towards even less glucose tolerance than the CO and Viv chow diets (Fig. 6A, B). A one-way ANOVA on AUC for different diets showed a main effect of diet ($F_{4,28}$=7.130, p<.0004). The reduced weight gain was not due to lower food consumption by the ST+CO mice since the mice on all the HFDs displayed similar food intake (Fig. 6C). Only the Viv Chow mice showed greater food consumption relative to HFD diets ($F_{15,60}$=2.2 p<.01 for time on diet and $F_{2.2,33}$=69, p<.0001 for diet). Taken together, we conclude that the obesogenic and diabetogenic component of conventional soybean oil is a compound other than stigmasterol. As noted previously, LA could be contributing to obesity but not to glucose intolerance (13).

Changes in oxytocinergic system due to dietary soybean oil are independent of stigmasterol

To determine whether stigmasterol affected *Oxt* expression, we performed RT-qPCR on RNA isolated from the hypothalamus of male mice fed the various diets (Fig. 7A). A one-way ANOVA showed a main effect of diet ($F_{3,14}$=4.82 p<.05, N=3-6/diet). Post-hoc analysis revealed lower *Oxt* expression in the stigmasterol-enriched diet (ST+CO) compared to either of the two soybean oil diets (*P*<.05). In contrast, Pfaffl

ratios for *Oxt* transcript levels were significantly up-regulated in PL+CO (*P*<.05) but were just below the level of significance in SO+CO (*P=.06*) relative to CO, consistent with the RNA-seq results.

Blood drawn at the time of sacrifice was used to measure plasma levels of oxytocin using an EIA (Fig. 7B). One-way ANOVA showed main effects of diet ($F_{4,24}$=12.84, p<.0001, N=3-10/diet). Post-hoc group comparisons revealed that plasma OXT in SO+CO (P<.05) and PL+CO diets (P<.001) was significantly elevated compared to CO, consistent with the notion that these changes are likely due to a component of soybean oil. That component, however, was not stigmasterol, as the ST+CO-fed mice had similar levels to those in the CO diet group but significantly lower plasma OXT compared to the PL+CO group (*P*<.001).

An analysis was done to correlate gene expression and plasma levels of oxytocin with body weight and glucose tolerance (GTT) in each of the dietary groups (Supplementary Fig. 3A) (46). The only correlation that we found to be significant was the *Oxt* mRNA versus GTT (r =.9, *P*=.04). The plasma OXT versus GTT was trending towards significance (r =.8, *P*=.06). In contrast, there were no significant correlations with body weight. Similarly, a heatmap of individual body weights of mice used for RNA-seq analysis did not show the same pattern as the gene expression profiles in Fig. 2A (Supplementary Fig. 3B) (46).

Central and peripheral oxytocin levels are dysregulated by components of soybean oil

To determine the abundance of OXT protein in the brain, hypothalamic tissue sections were incubated with an antibody that recognizes oxytocin-neurophysin, a carrier protein for OXT: cell bodies, dendrites and axonal projections of OXT-positive

neurons were observed in the SON and PVN of the hypothalamus (Fig. 8). Within the PVN and SON both MNCs (with cell body size >25 μm) and parvocellular neurons (cell bodies <25 μm) are intensely stained in Viv chow and CO-fed groups. In contrast, the density of those networks and the intensity of the signal was markedly decreased in the other HFDs: SO+CO, PL+CO and ST+CO. A total of seven immunohistochemical experiments were performed using 4-6 mice/group with similar results.

Discussion

The consumption of soybean oil, one of the most ubiquitous components of the American diet, has increased dramatically over the last several decades and parallels the increase in prevalence of both obesity and diabetes in humans (10,18). We and others have shown previously that HFDs enriched in soybean oil induce both obesity and diabetes in rodent systems (13–15). In the present study, we show that diets enriched in either conventional soybean oil high in LA (SO+CO) or a genetically modified soybean oil low in LA (and high in oleic acid), Plenish (PL+CO), made male mice more glucose intolerant compared to the control HFD, CO. In addition, the SO+CO diet caused more weight gain over the 16-week study than the other HFDs. Importantly, both the soybean oil-rich diets produced a considerable dysregulation of gene expression in the hypothalamus of male mice, the most notable of which is the gene coding for oxytocin (*Oxt*). We also observed elevated levels of circulating OXT peptide in the plasma of soybean oil-fed mice. An isocaloric coconut oil diet did not impact the expression of oxytocin either in the brain or the circulation, and had a minimal effect on hypothalamic gene expression compared to the low-fat control diet. Furthermore, the level of neither linoleic acid (LA) nor stigmasterol in the diets had a significant effect on

hypothalamic *Oxt* mRNA or plasma OXT. All told, our results demonstrate that different dietary oils can have differential effects on hypothalamic gene expression and raise the possibility that the soybean oil-rich American diet may be not only contributing to increased rates of metabolic disease but also impacting neurological function.

Chronic SO+CO and PL+CO diets produced similar hypothalamic transcriptomic profiles and dysregulated more genes than CO when compared to the low-fat control Viv Chow diet (Fig. 2A). While this suggests that components other than LA may be responsible for the majority of the effects on the hypothalamus, some subtle differences were noted between the low-LA and high-LA diets. The pathways altered by SO+CO include neuroendocrine, inflammation and insulin signaling and those altered by PL+CO include growth factor signaling. Neurochemical signaling pathways for opioid prodynorphin and vasopressin synthesis were common to both due to two genes: *Oxt* and *Avp*.

Impact of soybean oil diet on oxytocinergic system

Oxytocin signaling within the central nervous (CNS) and oxytocin hormone secretion into the periphery play important roles in regulating energy balance, body weight gain and glucose homeostasis (2,69,70). Central administration of OXT reduces diet-induced obesity and antagonism of central OXT receptors is obesogenic (71). In particular, *central* oxytocin within the PVN, an area critical for energy homeostasis and susceptible to the effects of HFD, can influence body weight regulation as has been shown for oxytocin and other hypothalamic neuropeptide systems previously (72,73). The role of peripheral oxytocin in regulating obesity and diabetes is more controversial as some studies report that circulating OXT can decrease fat mass and glucose

intolerance (74) while others find a positive association between plasma OXT and body mass index and glucose intolerance in humans (70,73,75,76). What is clear is that the metabolic functions of the oxytocinergic system are complex and dependent on both *central* and *peripheral* actions.

In the current study, HFDs enriched in conventional soybean oil (SO+CO) and low-LA Plenish (PL+CO) had effects on both the peripheral and central components of the oxytocinergic system. Plasma oxytocin peptide levels were increased in the blood: 250% (PL+CO) and 200% (SO+CO) relative to both the high-fat coconut oil (CO) and low-fat control (Viv chow) diets. While *Oxt* transcript levels in the hypothalamus were also significantly increased in both soybean oil diets, immunohistochemical experiments showed decreased OXT immunoreactivity in parvocellular cells and MNCs of the SON and PVN. This suggests a potential impact on central oxytocin release from MNC dendrites and synaptic neurotransmission as well as peripheral release of OXT originating in MNC axons that project to the posterior pituitary and release OXT into the systemic circulation (3,4). Others have shown that a chronic HFD (composition unspecified) results in elevated *Oxt* mRNA in the murine hypothalamus but blunts the stimulated release of central OXT from PVN slices manipulated *ex vivo*, and suggested that the increased *Oxt* transcription could be a compensatory mechanism for suppressed OXT release (77).

Regardless of which aspect of oxytocin function – central or peripheral – is more relevant to the metabolic effects of soybean oil, what is clear is that LA does not appear to play a role. Mice fed conventional soybean oil, high in LA (~55%), or the genetically modified Plenish, low in LA (7.4%), displayed essentially identical effects on every

aspect of oxytocin examined – elevated RNA levels in the hypothalamus, low protein immunoreactivity in SON and PVN and high peptide levels in plasma. While others have reported that PUFAs from various dietary sources (e.g., safflower and sunflower seed oils that are also high in LA) induce obesity in rodents (16,23,24), they did not examine changes in *Oxt.* They did report, however, an increase in expression of other hypothalamic neuropeptide genes such as *POMC*, *GALP*, *MCH*, *preORX* , *Agrp* and *Cart* (also known as *Cartpt)* as well as changes in pro-inflammatory genes *Akt, Ikk, IL6, Icam1 and Cx3cl1*. We did not observe a change of expression in any of these genes with either soybean oil diet, with the exception of *Cartpt*, *Pmch* (precursor to melanocortin concentrating hormone, MCH) and *Akt1s1,* a substrate of AKT, reinforcing the notion that the oxytocin-related changes observed in the soybean oil diets are likely due to some component other than LA.

Like LA, the addition of stigmasterol to the coconut oil diet (ST+CO) did not increase the levels of *Oxt* RNA in the hypothalamus nor OXT peptide in the plasma. However, the mice fed the ST+CO diet did show light hypothalamic OXT immunoreactivity, as did the two soybean oil diets, suggesting that stigmasterol could be affecting the release of OXT from the oxytocinergic neurons. Finally, it is worth noting that while we have examined only male mice in this study, the effects of soybean oil diets on female hypothalamus still needs to be addressed in future studies. We do not *a priori* expect sex differences produced by dietary soybean oil on solely this basis because soybean oil is free of phytoestrogens, such as isoflavanoids (78). However, since the hypothalamic oxytocinergic system shows sexual dimorphism, especially with

regard to maternal behavior, there may be potential sex differences resulting from dietary soybean oil.

Correlation between oxytocin gene and peptide expression and body weight and glucose intolerance in soybean oil diets

An alternative hypothesis to the soybean oil diets directly impacting the oxytocinergic system is that they exert effects on peripheral organs such as liver, adipose and muscle, causing obesity and diabetes, and that it is those metabolic states that impact *Oxt* expression rather than the constituents of the diets, or their metabolites, *per se.* Correlation analysis between body weight gain and glucose intolerance versus hypothalamic *Oxt* mRNA and circulating OXT peptide shows that while the obese state *per se* is not significantly correlated with *Oxt* levels, the diabetic state is (as measured by glucose intolerance). Indeed, others have reported that both spontaneous and induced diabetes results in an increase in OXT protein, although without changes in *Oxt* mRNA [5]. Also arguing against the notion that it is the diabetic state and not the diets directly that alter the oxytocinergic system, is the fact that the Plenish diet does not lead to another facet of diabetes, insulin resistance, while conventional soybean oil does (13). Nonetheless, if some aspect of the metabolic state does, in fact, impact *Oxt* expression, then the question arises about the nature of the underlying cause(s) of the changes in expression of non-metabolic-associated genes observed in the SO+CO and PL+CO diets (Fig. 2A). Finally, it is worth noting that while oxytocin is well known to regulate body weight, in part, by influencing eating behavior and satiety (2,71), a significant difference in the amount of food consumed between the different diets was not observed (Fig. 6C).

Impact of soybean oil diet on other genes linked to metabolic disease

In addition to *Oxt* there were several other genes related to metabolic function that were dysregulated by the soybean oil diets. Most notable of those was the neuropeptide vasopressin (*Avp*) which, like oxytocin, is expressed primarily in the SON and PVN MNCs and associated with body weight regulation and diabetes (73,79) and was upregulated in the hypothalamus of the SO+CO and PL+CO diets. Peripheral AVP has been recognized as a risk factor for Type 2 Diabetes (80), but the role of hypothalamic AVP in diabetes is still being explored (79).

Other genes commonly dysregulated in both soybean oil diets linked to metabolic disorders include *Cartpt, Hcrt, Mmp11, Abhd8* (all upregulated) (Fig. 5C). *Cartpt* (cocaine- and amphetamine-regulated transcript) gives rise to bioactive neuropeptides that are associated with food intake, body weight maintenance, and energy balance [81–83]. *Hcrt* encodes hypocretin, a hypothalamic neuropeptide precursor protein that gives rise to orexin neuropeptides which regulate feeding behavior, glucose metabolism, and homeostasis (84). The remaining two dysregulated genes – *Mmp11* and *Abhd8* -- have not been associated with activities in the brain but have been implicated in diabetes (*Mmp11*) (85,86) and lipid metabolism (*Abhd8*) (87).

Chronic HFD feeding can lead to diabetes and obesity via activation of pro-inflammatory pathways in the hypothalamus (7,8,88). While the two soybean oil diets (SO+CO, PL+CO) resulted in changes in several pro-inflammatory genes versus the coconut oil diet, the latter diet resulted in the increased expression of just two genes linked to inflammation – *Atp2a1* and *Rgs16* (Table 5). *Rgs16* has a prominent role in the trafficking of B and T lymphocytes and macrophages (89) but in the hypothalamus may

have a role in circadian regulation and food intake (90). Five of the nine genes dysregulated by both SO+CO and PL+CO versus CO and associated with both metabolic and neurological disorders are linked to inflammation (*Nr4a2*, *Ghsr*, *Oxt, Dio2, Mt1)*. These genes are different from the ones reported by others: *IKKbeta*, *NF-kB* and *c-Jun N-terminal kinase* (JNK) (7,8) , which could be due to the fact that the diets used in those studies were lard-based and contained a mix of saturated and unsaturated fats.

Impact of soybean oil diet on other genes linked to neurological disease

In addition to being linked to metabolic disorders, oxytocin signaling is associated with neurological disorders and behavioral and psychiatric functions (91). For example, low levels of oxytocin in the brain are related to depression (92), schizophrenia (93), pain and autism (92,94) and oxytocin treatment targeting both the central and peripheral systems has been shown to help ameliorate some of these conditions (95,96). Could low protein levels of oxytocin observed in the hypothalamus of mice fed the soybean oil diets potentially be linked to neurological dysfunction?

The results of two recent studies support the notion that HFD affects some aspect of central OXT signaling and reveal the behavioral implications of such an effect. In the first study, reduced OXT levels in the prefrontal cortex of rats fed a HFD were associated with reduced functionality of oxytocin signaling, namely impaired social preference, memory and synaptic potentiation, effects that were normalized by prefrontal injections of an OXT agonist (97). In the second study, a maternal HFD (lard-based, high in PUFAs as well as saturated fats) reduced the density of oxytocin-

immunoreactive neurons in the hypothalamus and negatively impacted “oxytocin-relevant” social behavior in offspring (98). Thus, it is conceivable that soybean oil diets resulting in a decrease in the central actions of oxytocin could play a role in modulating neurological function and behavior.

In addition to oxytocin, several other genes associated with neurological diseases were also dysregulated in both the soybean oil diets but not the coconut oil diet (Table 6). The upregulated genes include: *Avp,* associated with schizophrenia (99) and depression (100,101) (Fig. 4C); *Hcrt,* related to anxiety, depression and pain (102,103) (Fig. 5B); *Pcsk1n*, which is predictive of Alzheimer’s disease via association with the amyloid anti-aggregant protein, proSAAS (104) (Fig. 2C). In contrast, *Ankk1*, which has a role in dopamine receptor signaling was downregulated but only in the Plenish diet (Fig. 3B). Notably, all of these changes would lead to impaired neurological function. Finally, as we observed for oxytocin signaling, in addition to changes in gene expression, future studies will need to take into account changes in the neurotransmitter peptide levels and release. For example, *Kcng1*, the potassium voltage-gated channel modifier which is the only gene with a significantly different level of expression between SO+CO and PL+CO (Fig. 2C), belongs to a family of proteins that regulate neurotransmitter release, suggesting that other neurological signaling systems could be implicated, even if transcript levels are not.

The results presented here demonstrating the impact of dietary soybean oil on gene expression in the hypothalamus lead to the provocative suggestion that dietary fat, in general, and soybean oil, in particular, may have an impact on mental as well as metabolic health. The results also clearly indicate that additional studies are needed to

determine the effects of both high-LA and low-LA soybean oil on hypothalamic, and potentially other brain function and underscore the need for a careful evaluation of the extensive use of soybean oil-based food products, including infant formula, animal feed and other processed foods.

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Availability of data and material

Entire processed data is available on Figshare (see references 37 and 46).

Figure Legends

Figure 1. Study design and 3D-PCA showing differential effects of dietary fat on hypothalamic gene expression. A. Workflow showing the two cohorts of mice used, different diets, time on diets and various analyses performed. **B.** Principal components analysis (PCA) of RNA-seq data from hypothalami of male mice fed Viv chow, CO, SO+CO or PL+CO for 24 weeks showing three biological replicates for each diet. As indicated, the two soybean oil diets, SO+CO and PL+CO, can be grouped together as well as Viv chow and CO. GTT, glucose tolerance test; RT-qPCR, quantitative polymerase chain reaction; EIA, enzyme immunoassay; IHC, immunohistochemistry; Viv chow, Vivarium chow (low-fat control); CO, coconut oil diet; and

CO diets enriched in conventional soybean oil (SO+CO), genetically modified Plenish (PL+CO) and stigmasterol (ST+CO).

Figure 2. Soybean oil impacts the hypothalamic transcriptome. **A.** Heat map of differential hypothalamic gene expression (RNA-seq) between mice fed Viv chow, CO, SO+CO and PL+CO diets for 24 weeks. Color bar indicates level of gene expression relative to the whole data-set. **Top**, genes up-regulated in Viv chow versus the three HFDs; **Bottom**, genes downregulated in Viv chow. Arrows, genes mentioned in the text. Asterisk, *Kcng1,* the only gene significantly different between SO+CO and PL+CO. **B.** Gene ontology showing categories of molecular function affected by diet. **C.** Absolute expression levels from RNA-seq of indicated diets. DEG, differentially expressed genes; RPKM, reads per kilobase per million. Statistically different from $^{\#}$ CO, * Viv chow, $^{@}$ PL+CO.

Figure 3. Comparison of differentially expressed hypothalamic genes in different diet groups. A. Venn diagram of number of genes dysregulated in SO+CO (*left)* and PL+CO (*right)* versus CO and Viv chow diets ($\geq$ 1.5 fold, *P* and *padj* $\leq$.05). **B.** Absolute expression levels from RNA-seq of genes that are most dysregulated between SO+CO, PL+CO or both soybean oil diets versus CO. Statistically different from $^{\#}$ CO, * Viv chow.

Figure 4. Biological pathways associated with hypothalamic **genes dysregulated by soybean oil-rich HFDs. A.** Venn diagram showing number of genes dysregulated ($\geq$ 1.5 fold, *P* and *Padj* $\leq$.05) either exclusively or commonly by SO+CO and PL+CO versus CO and gene ontology for biological pathways altered in the comparisons shown. **B.** Absolute expression levels from RNA-seq of dysregulated genes ($\geq$ 1.5 fold, *P* and *padj* $\leq$.05) in the most prominent pathways. Significantly different from $^{\#}$ CO, * Viv chow.

Figure 5. *Oxt* is the only gene dysregulated in both soybean oil diets (vs CO and Viv chow) that is associated with neurological, metabolic and inflammation disease categories. A. Venn diagram showing overlap in dysregulated hypothalamic genes from RNA-seq related to neurological diseases versus metabolic diseases and inflammation dysregulated in SO+CO and PL+CO versus CO (Tables 5 and 6). **B.** Absolute expression levels from RNA-seq data of six of the nine common genes. Significantly different from $^{\#}$ CO, * Viv chow. **C.** Venn diagram showing overlap in genes related to metabolic diseases (obesity, diabetes and lipid metabolism), inflammation and neurological diseases that are dysregulated in the hypothalamus of both soybean oil diets versus CO diet. Oxytocin (*Oxt*, red font) is the only gene that is common to all three disease categories and is significantly different from both CO and Viv chow.

Figure 6. Obesogenic and diabetogenic effects of soybean oil diets with high or low LA is not mimicked by stigmasterol. A. Average weekly body weights of male C57BL/6N mice started on the indicated diets at weaning (N=6-12/diet) as in Fig. 1A. All diets were isocaloric with 40 kcal% total fat except Viv chow which had 13.4 kcal% fat. **B**. Glycemia (mg/dL) measured during a glucose tolerance test (GTT) depicted as area under the curve (AUC) after 16 weeks on diet (N=6-12/diet). **C**. Average weekly food consumption of mice on various diets measured on a per cage basis and normalized to the number of mice per cage. Food was changed and measured twice weekly; values were combined to generate the weekly average. Consumption of Viv chow was highest because this diet has the fewest calories per gram (N=6-12 mice (3-4 cages)/diet). $^{@}$ indicates statistical difference between PL+CO and ST+CO at 12 weeks on diet. Statistically different from: $^{\#}$ CO, $^{\%}$ ST+CO, $^{\&}$ all others using one-way ANOVA followed by Tukey's post-hoc analysis for **B** or two-way ANOVA followed by Tukey's post-hoc analysis for **A, C.**

Figure 7. Dietary soybean oil dysregulates hypothalamic gene expression and plasma levels of oxytocin. C57BL/6N male mice fed Viv chow, CO, SO+CO, PL+CO or ST+CO diets for 17-28 weeks. **A.** Transc--ript levels of *Oxt*, expressed relative to beta-actin (*Actb)* measured in hypothalamic homogenates using RT-qPCR and expressed relative to levels in CO using the Pfaffl method (N=3-6/diet). **B.** Peripheral oxytocin peptide levels measured via EIA, (N=3-10/diet). Statistically different from: $^{\#}$ CO; $^{@@@}$ PL+CO; $^{\wedge}$ SO+CO and PL+CO (single symbols, *P* <.05; triple symbols *P*<.001) using one-way ANOVA followed by Tukey's post-hoc analysis for **A** and one-way ANOVA followed by Bonferroni's post-hoc analysis for **B**.

Figure 8. Dietary soybean oil decreases oxytocin immunoreactivity in the magnocellular neuroendocrine nuclei of the hypothalamus. Oxytocin-neurophysin immunoreactivity in micrographs of hypothalamic tissue sections from perfused brains of male mice fed Viv chow (Viv), CO, SO+CO, PL+CO or ST+CO diets for 17-28 weeks. Reduced immunofluorescence was observed in SO+CO, PL+CO and ST+CO compared to CO and Viv chow in the large magnocellular neuroendocrine and smaller parvocellular neurons of the SON and PVN. PVN = paraventricular nucleus; SON = supraoptic nucleus; 3V = third ventricle; OX = optic chiasm. Arrow = magnocellular neuroendocrine cell; Arrowhead = parvocellular neuron; Asterisk = immunolabeled axonal projections; dashed line=PVN boundary in SO+CO and ST+CO; Calibration bar =100 µm.

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Table 1. Composition of diets and oils used in this study

Nutrient composition (gm%) of the diets					
	Viv Chow*	**CO**	**SO+CO**	**PL+CO**	**ST+CO**
Protein	23.9	20.1	20.1	20.1	20.1
Carbohydrate	48.7	53.4	53.4	53.4	53.4
Fat	5	21.5	21.5	21.5	21.5
kcal/gm	3.36	4.87	4.87	4.87	4.87
Fat (kcal%)	13.4	40	40	40	40
Source of Fat (gm%)	**Viv Chow***	**CO**	**SO+CO**	**PL+CO**	**ST+CO**
Porcine Animal Fat	4.5	0	0	0	0
Soybean Oil	0	25	115	0	25
Plenish Oil	0	0	0	115	0
Coconut Oil, Hydrogenated	0	220	130	130	220
Sterols (gm/1.1kg)					
Stigmasterol	0	0	0.1	0.1	0.1

* Purina Test Diet 5001

Table 2. Fatty acid composition of oils used in this study

Fatty acid (%)	Coconut	Soybean	Plenish
Lauric (12:0)	45	<0.05	<0.05
Myristic (14:0)	17.5	0.07	<0.05
Palmitic (16:0)	8.67	10.6	5.81
Stearic (18:0)	10.2	3.98	4.17
Oleic (18:1)	0.25	20.9	73.9
Linoleic (18:2ω6)	<0.06	52.9	7.42
α-Linolenic (18:3ω3)	<0.06	6.54	1.91
ω6:ω3 (18:2/18:3)	0	8.1	3.4

Composition was determined by Covance Labs.
No isoflavones were detected in these oils.

Table 3. Phytosterol composition of oils used in this study

Phytosterol (mg/100gm)	**Coconut**	**Soybean**	**Plenish**
Campesterol	6.6	61.1	61.1
Stigmasterol	9.1	65	64
b-sitosterol	44.9	177	131
Brassicasterol	<1	<1	<1
Other Sterols	24.7	31.6	23.9
Total Sterols	85.3	335	280

Composition was determined by Covance Labs.
No isoflavones were detected in these oils.

Table 4. RT-qPCR primers used in this study

Primer	Accession #	Sequence Forward 5'-3' Reverse 5'-3'	T_m °C (forward/ reverse)	Product length (bp)	Primer efficiency(%)	Exon target (forward/ reverse)
Oxt	NM_011025.4	TTGGCTTACTGGCTCTGACCTC GGGAGACACTTGCGCATATCCAG	62.0/63.4	97	101.8	1/2
Actb	NM_007393(1)	GACTCATCGTACTCCTGCTTG GATTACTGCTCTGGCTCCTAG	61.9/61.3	147	101.1	5/6

Abbreviations: *Oxt*, oxytocin; A*ctb,* b-actin; bp, base pair; Tm, melting temperature.

Table 5. Dysregulated hypothalamic genes related to metabolic disease and inflammation in male mice (*P*≤0.05; q≤0.05; FC ≥1.5).

Diet	Metabolic Disease			
	Diabetes	**Lipid Metabolism**	**Obesity**	**Inflammation**
SO+CO vs CO	*Oxt , Mmp11, Hcrt, Avp, Hsd11b1, Gck, Hdc, Cartpt*	*Hsd11b1, Ghsr, Gck, Abhd8, Hsd17b7*	*Mc3r, Hsd11b1, Hcrt, Ghsr, Gal, Gck, Cartpt, Gpx3, Dio2*	*C1qtnf4, Oxt, Hsd11b1, Ghsr, Cartpt, Nr4a2, Dio2, Sema7a*
PL+CO vs CO	*Hcrt, Mmp11, Oxt, Avp, Cartpt, Mt1*	*Abhd8*	*Hcrt, Cartpt, Mt1*	*C1qtnf4 , Oxt, Crlf2, Cartpt, Mt1*
CO vs Viv	0	*0*	0	*Atp2a1, Rgs16*

Genes are presented in order of decreasing fold-change.

Table 6. Dysregulated hypothalamic genes related to neurological disease in male mice ($P \leq 0.05$; $q \leq 0.05$; FC ≥ 1.5).

Diet	Alzheimer's	Anxiety	Autism	Depression	Pain	Parkinson's	Schizophrenia
SO+CO vs CO	*Pcsk1n, Gal, Irs4 ,Ngb, Nrn1*	*Oxt, Hcrt, Avp, Gal, Hdc,Dio2, Baiap3*	*Oxt*	*Oxt, Hcrt, Avp, Gal, Shisa6, Dio2*	*Oxt, Hcrt, Ghsr Gal, Prkcg*	*Ghsr, Nr4a2*	*Nr4a2*
PL+CO vs CO	*Pcsk1n*	*Hcrt, Oxt, Avp*	*Oxt, Nrxn2*	*Hcrt, Oxt, Avp, Mt1*	*Hcrt, Oxt*	*0*	*Nrxn2*
CO vs Viv	*0*	*0*	*0*	*0*	0	*0*	*0*

Genes are presented in order of decreasing fold-change.

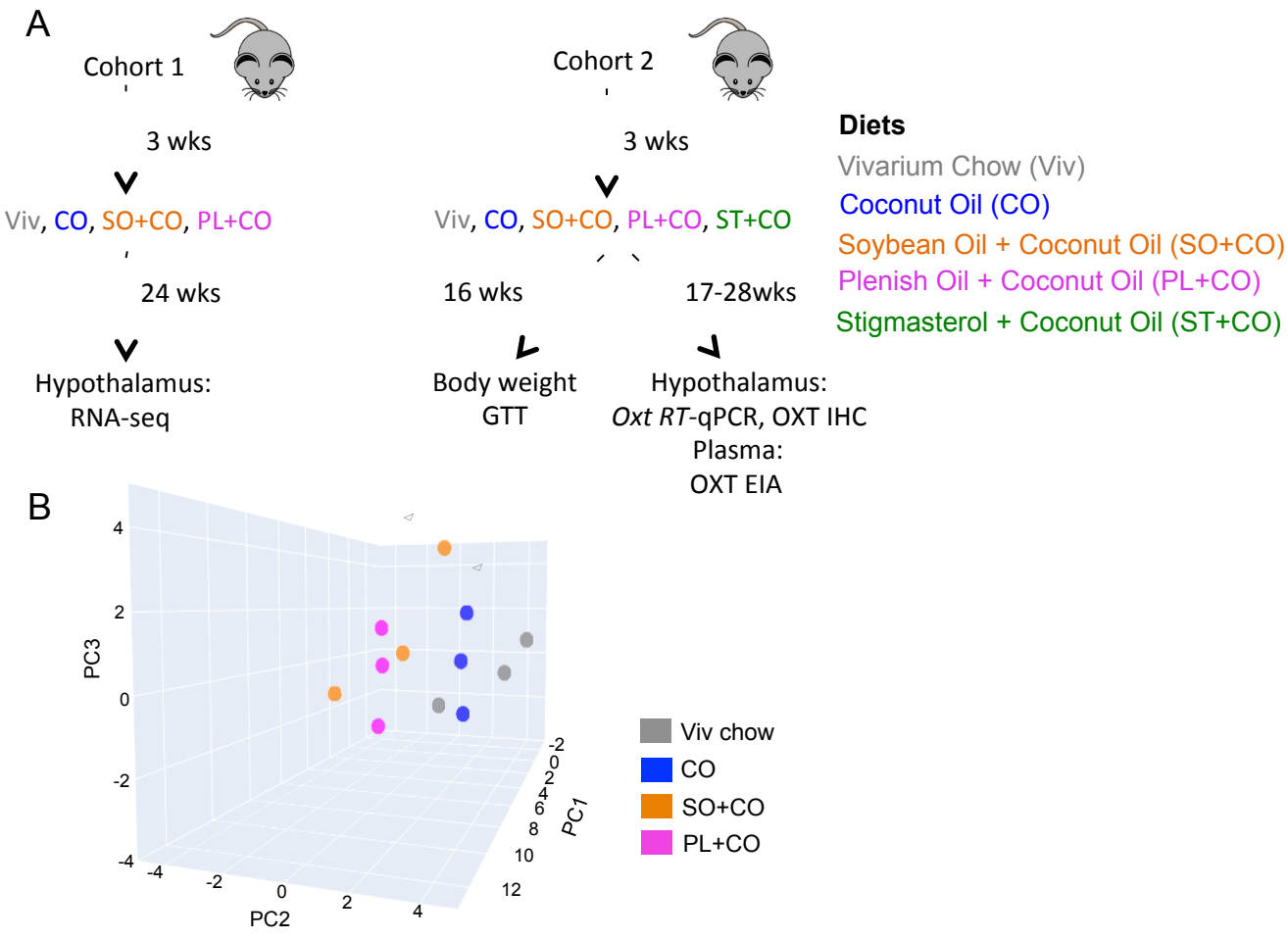


Figure 1

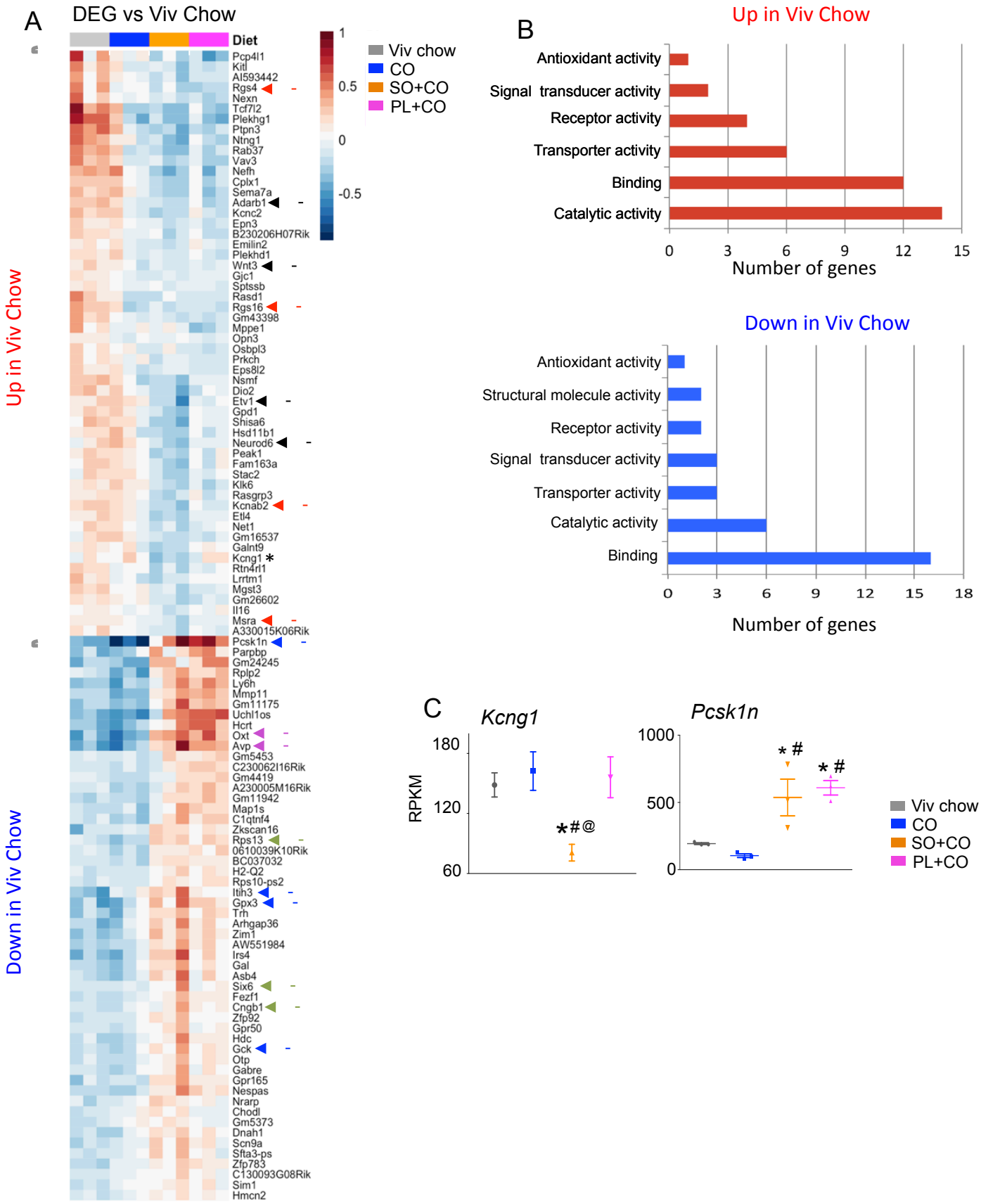


Figure 2

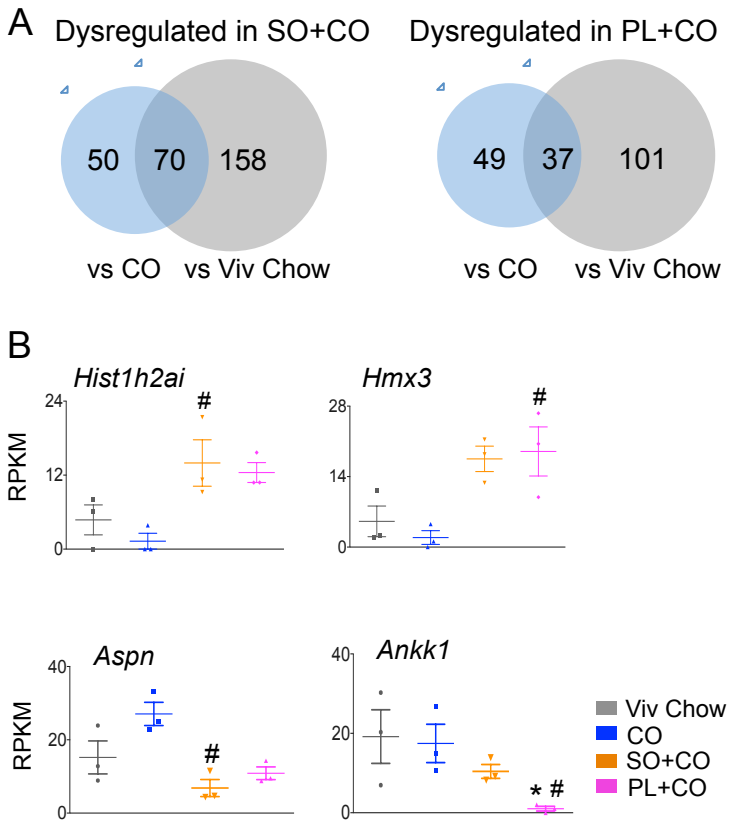


Figure 3

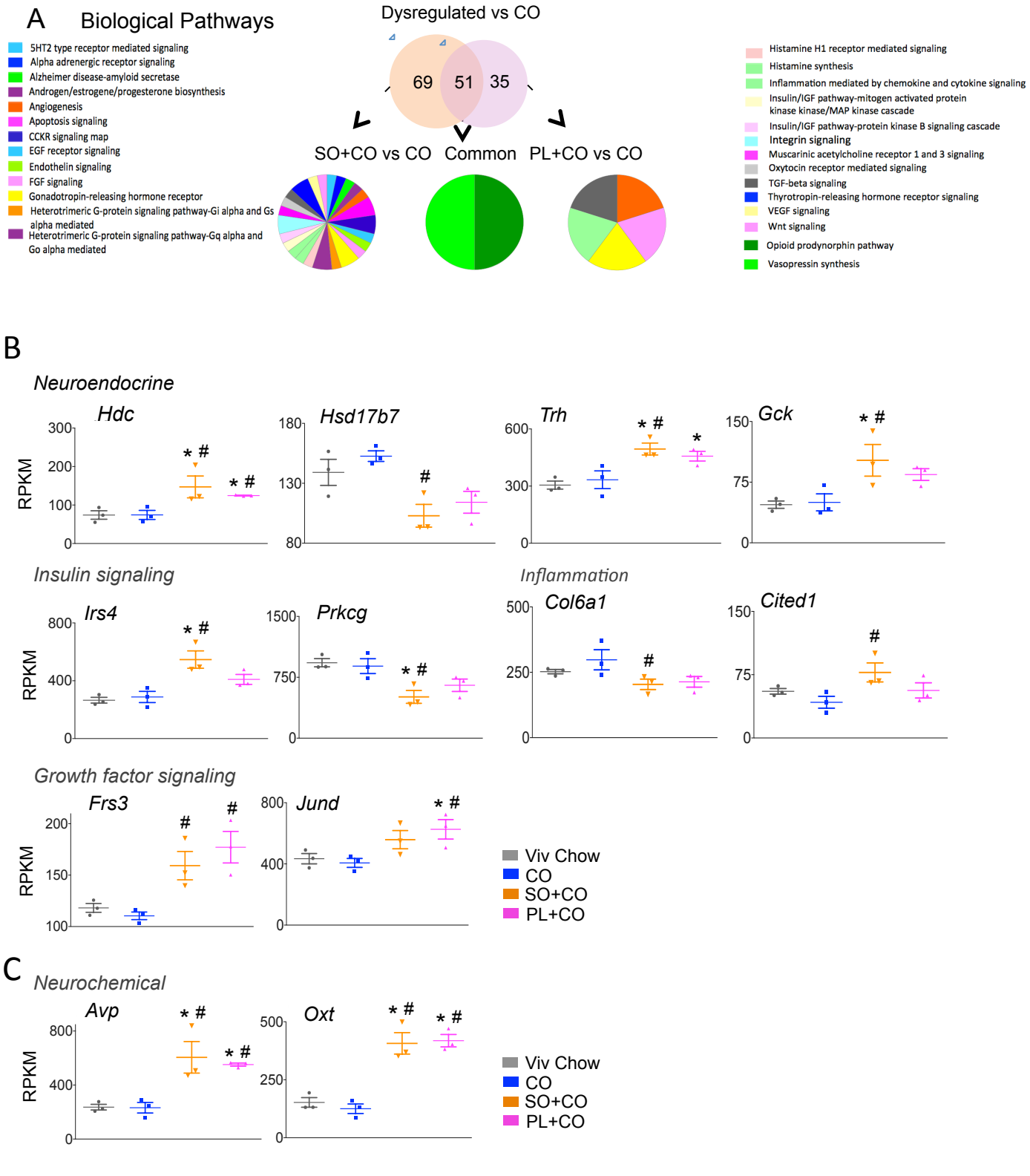
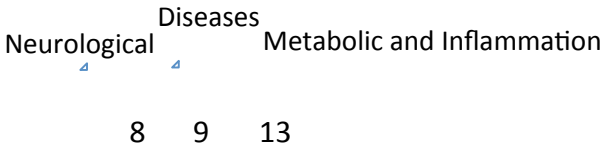
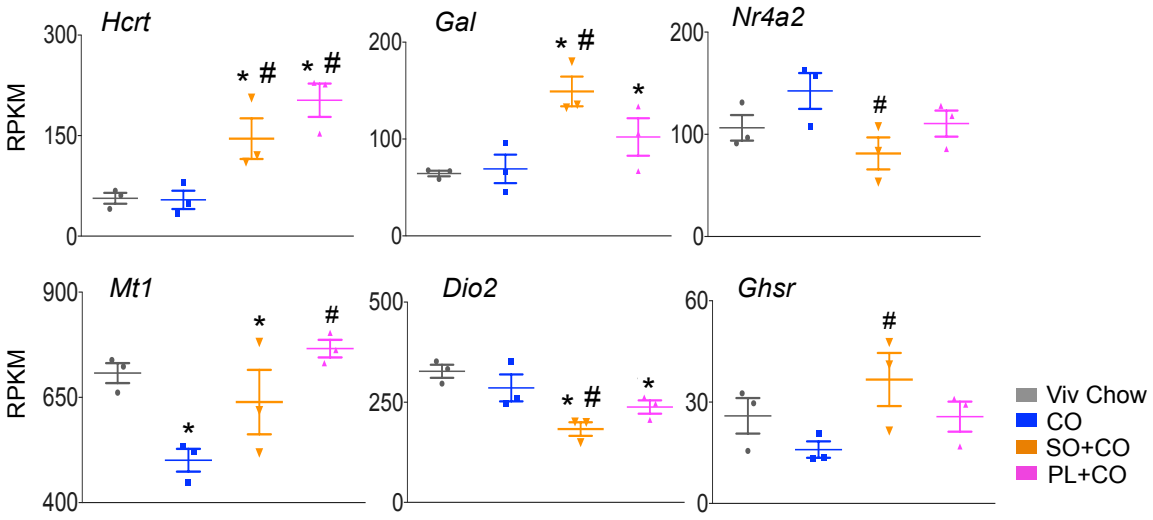


Figure 4

A



B



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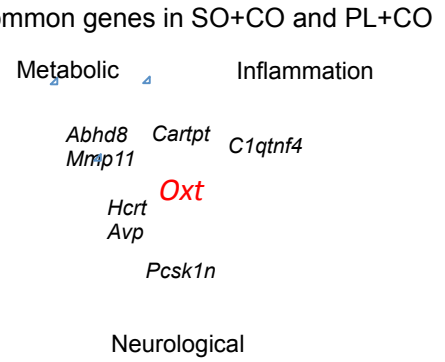


Figure 5

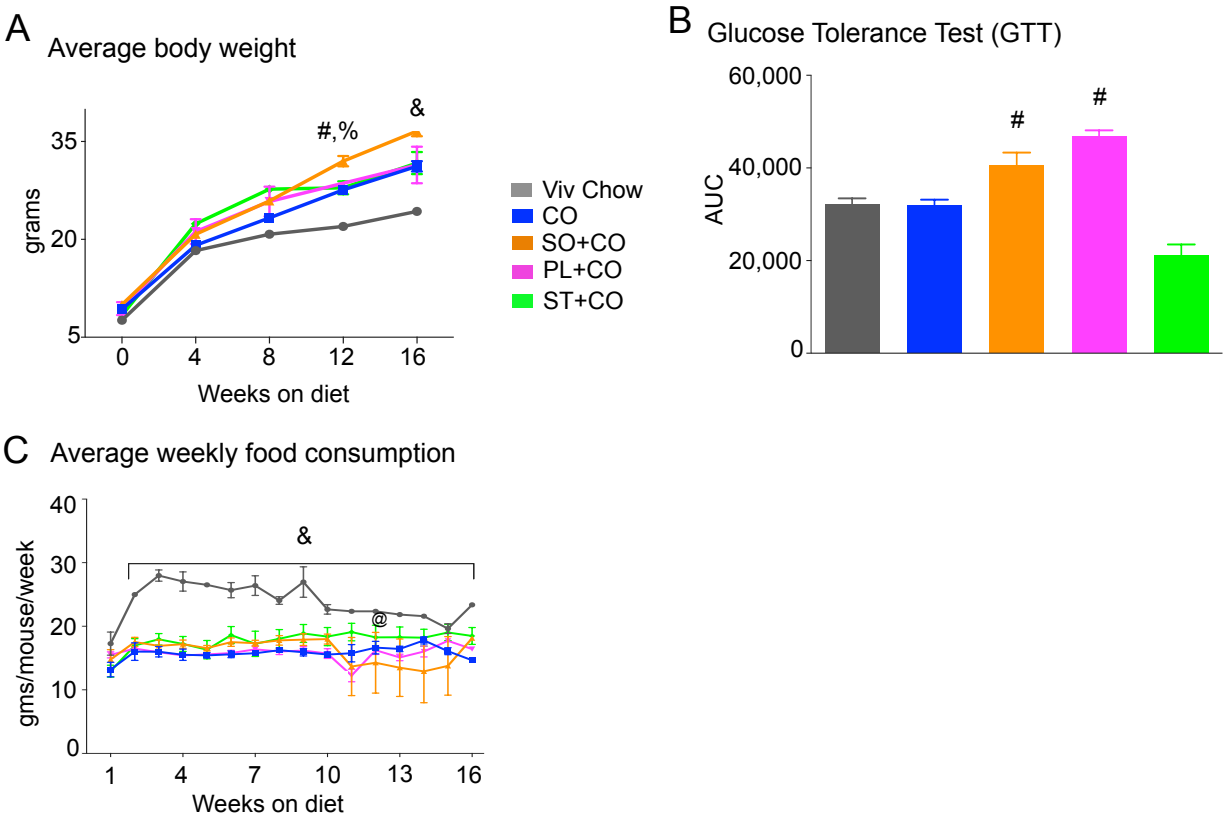


Figure 6

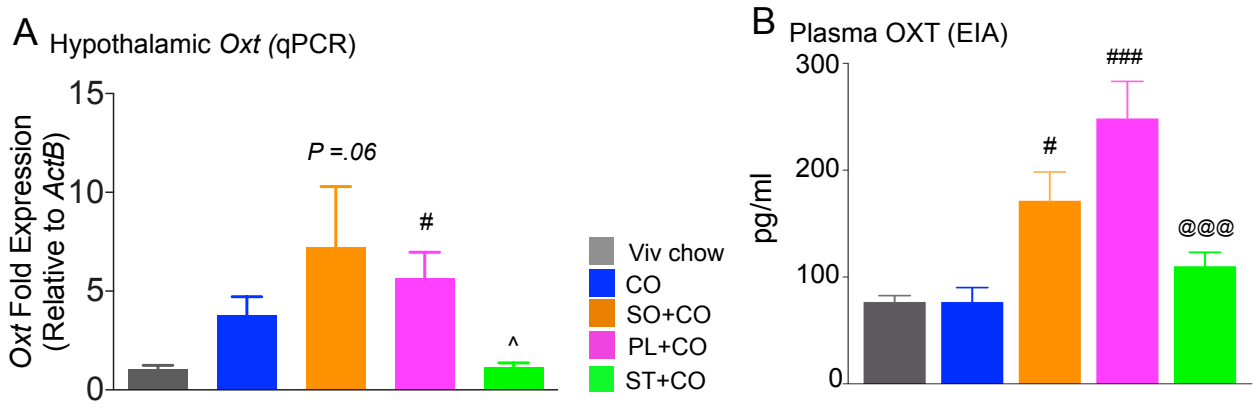
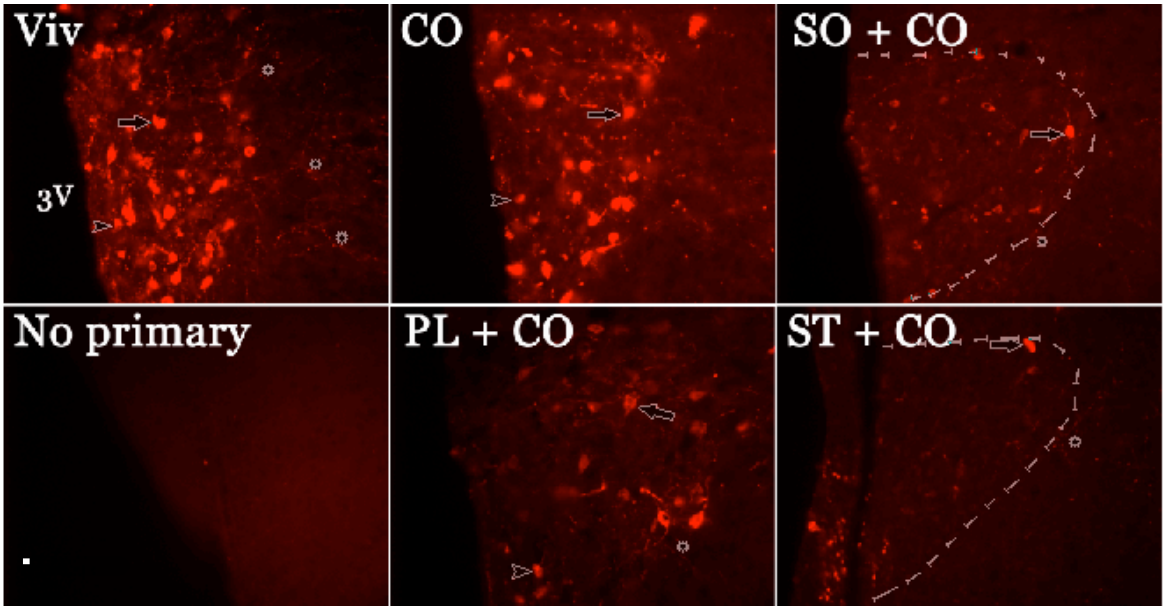


Figure 7

PVN



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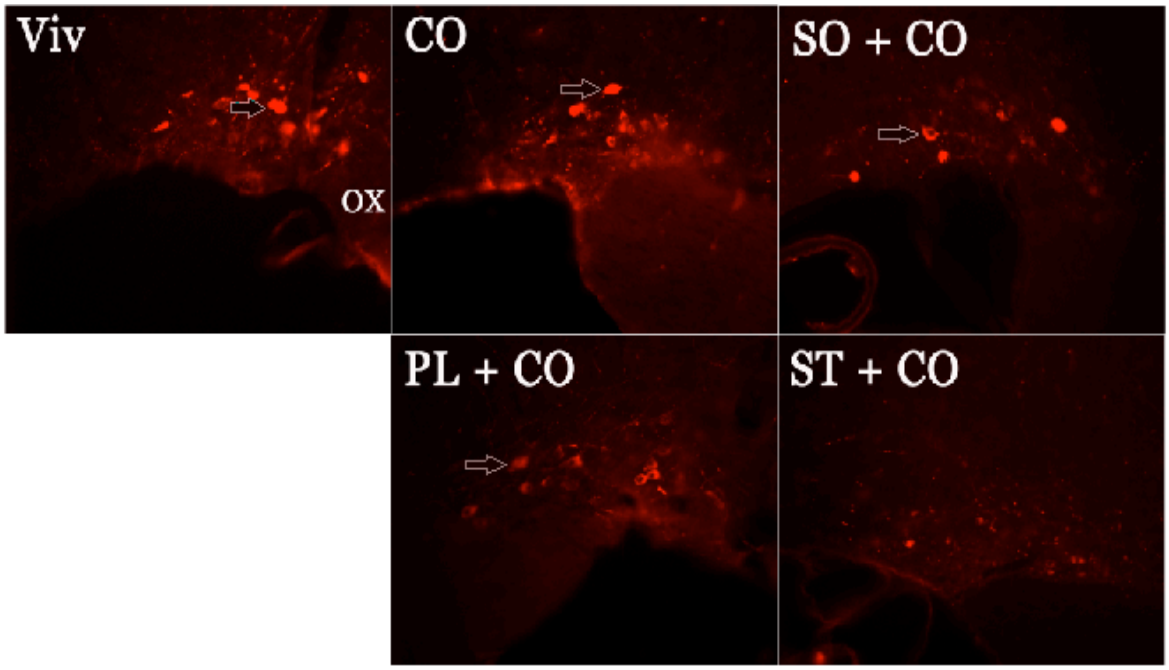

Figure 8