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Journal

Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1869(6)

ISSN

0925-4439

Authors

Khandelwal, Mayuri
Krishna, Gokul
Ying, Zhe
et al.

Publication Date

2023-08-01

DOI

10.1016/j.bbadis.2023.166728

Peer reviewed

Published in final edited form as:

Biochim Biophys Acta Mol Basis Dis. 2023 August ; 1869(6): 166728. doi:10.1016/j.bbadis.2023.166728.

Liver acts as a metabolic gate for the traumatic brain injury pathology: Protective action of thyroid hormone

Mayuri Khandelwal[a], **Gokul Krishna**[a], **Zhe Ying**[a], **Fernando Gomez-Pinilla**[a,b,*]

[a]Department of Integrative Biology and Physiology, University of California, Los Angeles, CA, USA

[b]Department of Neurosurgery, UCLA Brain Injury Research Center, Los Angeles, CA, USA

Abstract

Clinical evidence indicates that injury to the brain elicits systemic metabolic disturbances that contributes to the brain pathology. Since dietary fructose is metabolized in the liver, we explored mechanisms by which traumatic brain injury (TBI) and dietary fructose influence liver function and their possible repercussions to brain. Consumption of fructose contributed to the detrimental effects of TBI on liver operation, in terms of glucose and lipid metabolism, *de novo* lipogenesis, lipid peroxidation. Thyroid hormone (T4) is metabolized in the liver and found that T4 supply improved lipid metabolism by reducing *de novo* lipogenesis, lipid accumulation, lipogenic enzymes (ACC, AceCS1, FAS), lipid peroxidation in liver in response to fructose and fructose-TBI. T4 supply also helped to normalize glucose metabolism and improve insulin sensitivity. Furthermore, T4 counteracted elevations of the pro-inflammatory cytokines, *Tnfα* and *Mcp-1* after TBI and for fructose intake in liver and circulation. T4 also exerted an effect on isolated primary hepatocytes by potentiating phosphorylation of AMPKα and AKT substrate, AS160, leading to increased glucose uptake. In addition, T4 restored the metabolism of DHA in the liver disrupted by TBI and fructose, adding important information to optimize the action of DHA in therapeutics. The overall evidence seems to indicate that the liver works as a gate for the regulation of the effects of brain injury and foods on brain pathologies.

Keywords

Fluid percussion injury; Fructose; Liver; Insulin signaling; Thyroid hormone; Lipid metabolism

*Corresponding author at: Department of Integrative Biology and Physiology, University of California, 612 Charles E Young Drive, South, Los Angeles, CA 90095, USA. fgomezpi@ucla.edu (F. Gomez-Pinilla).

CRediT authorship contribution statement

Mayuri Khandelwal: Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft. **Gokul Krishna:** Methodology, Data curation, Formal analysis, Writing - review & editing. **Zhe Ying:** Data curation, Resources. **Fernando Gomez-Pinilla:** Conceptualization, Supervision, Project administration, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2023.166728.

1. Introduction

Traumatic brain injury (TBI) is a prevalent medical problem affecting 1.5 million people annually in United States [1,2]. Most injuries occur during sport playing, traffic accidents and military operations. Although a growing number of clinical observations show strong peripheral abnormalities in TBI patients, mechanisms involved are poorly understood. Liver is a primary station and master regulator of detoxification. It plays important role in metabolism of nutrients, synthesis of proteins and lipids used by brain and body. There is a bidirectional communication between brain and periphery, in which brain and body interact with each other *via* the autonomic nervous system, hormones, and metabolites [3,4]. Recent studies show that alterations in brain pathogenesis results in liver steatosis, metabolic dysfunction, such that disturbances in peripheral tissues is a major concern for TBI patients.

Diet is an important conduit to influence peripheral and central metabolism [5–7]; however, the events that communicate peripheral with central metabolism are poorly understood, particularly, in the pathology of TBI. Fructose consumption produces a stage of metabolic dysfunction that reduces the recovery potential after TBI [7]. Fructose is found in soft drinks and processed foods, and liver is the main site for its metabolism, hence sustained fructose consumption can have a heavy toll in individuals who are metabolically unstable, including TBI patients. Therefore, it is essential to understand how TBI pathology and fructose consumption interact at the liver level. Overconsumption of fructose can desensitize the insulin receptors responsible for glucose disposal causing development of insulin resistance [8,9]. Subsequently, insulin resistance enhances *de novo* lipogenesis (DNL) leading to accumulation of fatty acids/triglycerides in the liver [10,11]. DNL can convert excess carbohydrate into fatty acids that are then esterified to store triacylglycerols that can be used as energy source. Additionally, since fructose intake disrupts membrane integrity, it is important to understand how fructose affects metabolism of the omega-3 fatty acid docosahexaenoic (DHA) in the liver [9]. DHA is an integral component of cell plasma membranes and crucial for maintaining membrane integrity [12] throughout brain and body.

Liver is a central site of interaction among ingested foods, hormones, and metabolites, and subsequent synthesis of lipids and proteins which are essential for brain and body. Thyroid hormone is critical for regulation of lipid, glucose, cholesterol metabolism and insulin sensitivity [13,14]. Thyroid hormone tightly regulates *de novo* lipogenesis *i.e.*, a process to convert glucose to fatty acids and maintains both the anabolic and catabolic states during lipid metabolism [15]. Patients suffering from hypothyroidism show increased levels of triglyceride and cholesterol in serum [16]. Interestingly, our single cell genomic data in mice showed that TBI affects the transthyretin gene (*Ttr*) in the hippocampus which encodes the transporter of thyroxin (T4) across the blood-brain barrier [17,18]. Since thyroid hormone is metabolized in the liver, we sought to determine how T4 could interact with the effects of fructose and TBI on the liver. We expect to generate leads for novel therapeutics using liver as a potential target to reduce TBI pathogenesis.

2. Material and methods

2.1. Animals and experimental design

Male Sprague-Dawley rats (10 weeks old; 250 ± 20 g) were purchased from Charles River laboratories, Inc., MA, USA. Rats were group housed in polyacrylic cages and maintained under standard housing conditions in controlled environment (room temperature 22–24 °C and humidity) with 12 h light/dark cycle and acclimated for 7-days prior to experimentation with *ad libitum* access to food and water. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California at Los Angeles (UCLA) Chancellor's Animal Research Committee.

2.2. Experimental design and fluid percussion injury

Rats were randomized to regular or 15 % (*w/v*) fructose (Now Foods, Bloomingdale, IL) treatment for 21 consecutive days with free access to food. It has been previously established that consumption of 15 % fructose for 3 weeks is sufficient to develop peripheral metabolic abnormalities in rats [19]. We examined in the current study how fructose pre-treatment could impact TBI. Following drinking intervention, rats were subjected to either sham surgery or Suid percussion injury (FPI) as previously described [20]. BrieSy, rats were anesthetized with isoflurane (2.5 % in 100 % O_2) through nose cone using anesthesia system (Vet Equip Inc., CA, USA). After the surgical site was shaved, the head was secured in a stereotaxic apparatus and eye ointment was applied for corneal protection. The surgical site was then sterilized with alternating applications of iodine and 70 % ethanol. A midline incision was made over the skull, the skin and fascia were retracted. A 3-mm diameter craniectomy was made over the left parietal cortex (centered at the 3 mm posterior relative to bregma and 6 mm lateral to the midline) using a high-speed drill (Dremel, WI, USA) leaving the dura intact. A rigid plastic injury cap made from rigid luer-loc needle hub was placed and secured over the craniectomy site with dental acrylic cement. When the dental cement hardened, the anesthesia was discontinued. The hub was filled with non-pyrogenic saline and the hub was then attached to the FPI device. At the first sign of hind-limb withdrawal to a paw pinch, a moderate pulse of saline (approximately 2.7 atm) was imposed on the dura by releasing the pendulum onto the fluid-filled cylinder. Immediately after the injury, the response of each rat to a paw pinch was observed, followed by re-anesthetization, hub removal and skin suture. Antibiotic was applied and the rats were placed in a recovery chamber to be fully ambulatory before being returned to their cages. Carprofen (pain killer) was injected subcutaneously in rats 45 mins pre-TBI and 24 h post-TBI at the dose of 5 mg/kg for 3 days. Sham rats underwent identical surgical procedure as injured rats but without injury induction.

2.3. In vivo drug treatment

At 30 min post-surgery or FPI, rats received intra-peritoneal (i.p.) injection of either thyroid hormone (L-thyroxine sodium salt pentahydrate, T4, 10 μg/kg (Sigma Aldrich, Cat. No. T2501)), or an equivalent volume of non-pyrogenic saline for twelve consecutive days [21]. The dose was selected according to previous publications [17,18]. It has been reported that daily administration of T4 at a concentration of 20 μg/ml for two weeks increases

the serum T4 levels by 10 folds but only 1.2 folds in the hypothyroid brain [22]. We wanted to ascertain to have enough T4 in the hypothyroidic condition, which is the most common in patients and rodents after TBI. Therefore, T4 was administered daily in TBI rats with/without fructose consumption. Freshly prepared fructose in the drinking water was administered throughout the experimental duration. There were seven groups of eight animals as shown in Table 1. On day 11 post-TBI glucose tolerance test was performed and 24 h later, all rats were humanely euthanized, tissues were collected and stored at 80 °C for histological and biochemical studies (Fig. 1a). We aimed to perform GTT by the end stage (day 11 post-TBI) to study the effects of T4 after TBI and fructose consumption on glucose metabolism.

2.4. Fluid intake, food consumption and body weight measurements

Throughout the experimentation, two rats were kept per cage to reduce stress related to isolation [23]. Fructose in drinking water intake (ml/rat/day) and food consumption (g/rat/day) were measured daily up to 3 weeks to determine caloric intake. The total amount and volume of food/drink consumed was divided by two to identify the approximate amount consumed by each rat. For Suid intake, a constant volume (500 ml) of freshly prepared fructose dissolved in water (15 %) was added every morning and total volume consumed by rats was recorded prior to adding fresh fructose (15 %). For food measurements, each morning 500 g of food was added in each cage and the remaining food was weighed to determine the food intake by rats before adding fresh food. Body weight was measured throughout the study.

2.5. Glucose tolerance test (GTT)

We performed GTT by the end stage (day 11 post-TBI) to study the effects ofT4 after TBI and fructose consumption on glucose metabolism. Rats were fasted for 16 h prior performing glucose tolerance test, weighed and basal blood samples were taken from the tail tip. Rats were injected intraperitoneally with glucose (dextrose at 2 g/kg) and further blood samples were taken at 15-, 30-, 60- and 120-min using glucometer (Bayer's Contour, NJ, USA) and glucose levels were measured. The time for blood withdrawal was kept during 08:00–12:00 h to minimize diurnal variability. The area under the curve was calculated for each group.

2.6. Immunoblotting

Liver homogenates and cell lysate were prepared using lysis buffer with phosphatase inhibitor cocktail. Lysates were homogenized and centrifuged at 13,000 rpm for 30 min and the supernatant was collected. The total protein was then measured using BCA method (Pierce, ThermoFisher scientific, USA), and 25 μg protein was loaded on gel to perform PAGE and western blotting. The percentage of gel to be used was selected based on the molecular weight of the desired protein. Further, the membrane was blocked using 5 % BSA in TBST. Next, membrane was washed with 1× TBST 3 times followed by primary antibody overnight incubation at 4 °C. Bound antibody was visualized using HRP conjugated secondary antibody (1 h at room temperature) and membrane was developed using gel documentation instrument (Bio-Rad, USA) (Table 2).

2.7. Real time PCR

RNA was isolated from the liver using total RNA kit II (Omega BIO-TEK). First strand cDNA was synthesized from 1 μg RNA using c-DNA synthesis kit (applied biosystems, USA). Real Time PCR was performed from 10 ng cDNA using gene specific primers (Table 3). CFX-96 (Bio-Rad, USA) instruments were used to run the reaction using SYBR Green. The fold change was calculated using $2^{-\Delta\Delta Ct}$ method. The specific primers were designed for studying gene expression in liver.

2.8. Oil red O staining

The liver was isolated and cryoblocks were prepared. Sections of 8 μm were cut with cryostat and mounted on glass slides. For oil red O staining, slides were air dried followed by fixation with 10 % formalin for 5 min. Sections were rinsed twice with water for 5 min, and then rinsed with 60 % isopropanol for 30 s. The pre-warm oil red o stain was added on each section and incubated for 30 min at room temperature. After incubation, the slides were washed twice with 60 % isopropanol for 30 s each, and then rinsed with water two times for 5 min each. Lastly, slides were mounted and observed under 40× magnification using Leitz Apotome microscope and analyzed using Image J software.

2.9. Gas chromatography: fatty acid analysis

Total lipids were extracted from liver tissue according to the method of Bligh & Dyer [30]. Frozen livers were homogenized in chloroform/methanol (2:1 v/v), containing 50 μg/ml of butylated hydroxytoluene to prevent lipid oxidation during lipid isolation. Tricosanoic acid methyl ester (C23:0) as an internal standard. For lipid isolation, tissues were homogenized to powder under liquid nitrogen and subjected to extraction of total lipids. Fatty acid methylation was done by heating at 90 °C for 1 h with boron triSuoride-methanol reagent (14 % *w/v*). Extracted lipids were analyzed using Clarus 500 gas chromatograph (GC; PerkinElmer) with auto sampling function. An Elite-WAX column (60 m, 0.32 mm internal diameter, PerkinElmer) was used with hydrogen as the carrier gas. GC oven temperature was initially held at 140 °C for 2 min and raised with a gradient of 5 °C/min until 250 °C and held for 10 min. The injector and detector were maintained at 250 °C and 300 °C, respectively. The total run time for each sample was 34 min. Fatty acids were identified and quantified by comparison with standard peak (Supelco 37-component FAME Mix) and GLC reference standard 682 (NuCheck Prep, Inc. MN, USA).

2.10. Primary hepatocyte culture

Primary hepatocytes were isolated by retrograde perfusion. BrieSy, liver was perfused *via* vena cava with perfusion buffer (HBSS buffer without Ca^{2+}, Mg^{2+}, EDTA and HEPES) immediately after the portal vein canulation. Further, the digestion buffer containing HBSS buffer with Ca^{2+}, Mg^{2+} and HEPES was passed followed by liberase treatment. The liver was excised in digestion buffer followed by hepatocytes release in the suspension with the help of cell lifter. The isolated hepatocytes were then purified using percoll gradient centrifugation. Lastly, the cells were plated on the collagen treated culture dishes in plating media (low glucose DMEM). After 3 h cells were in maintenance media (Williams E) followed by treatments. The isolated hepatocytes were maintained in fructose enriched

medium overnight followed by exogeneous treatment with T4 (16 h, 100 nm). Primary cells were used to perform immuno-blotting and glucose uptake assay.

2.11. Glucose uptake assay in hepatocytes

Primary cells were grown in 12 well plates and after 80 % confluency, T4 was added. On completion of treatment for 16 h, cells were washed with buffer containing NaCl (140 mM), KCl (5 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1 mM), KH_2PO_4 (1 mM) and HEPES (10 mM) at pH 7.4 followed by 2 h incubation in the same buffer. Cells were rinsed again with the above-mentioned buffer and cells were incubated in 2-NBDG at 50 μM concentration (prepared in the same buffer) for 1 h at 37 °C in 5 % CO_2. Next, cells were lysed using lysis buffer containing sodium deoxycholate 1 % NP-40, KCl (40 mM) and Tris (20 mM) with pH 7.4 using cell scraper and centrifoged at 13,000 rpm at 4 °C for 20 min. Supernatant was collected and amount of total protein was estimated in each sample using BCA method. Fluorescence was measured in supernatants in duplicates using fluorescence reader (Tecan) at excitation and emission wavelength of 485 nm and 535 nm respectively. Finally, fluorescence was normalized with protein content of the respective sample [24,25].

2.12. Sandwich ELISA

The plasma concentration for Mcp-1 was performed according to the manufacturer's protocol (Boster, Cat. No. EK0902). BrieSy, for measurement of Mcp-1 in plasma, rats were euthanized after competition of treatment and blood was collected. The blood was stored at 4 ° C for 30 min followed by centrifugation at 2000 *g* for 10 min at 4 °C. The plasma and standards were added in the plate followed by addition of Bio-tinylated anti-rat Ccl2 antibody. Next, avidin-biotin peroxidase complex (ABC-HRP) was added. The color developing solution, TMB (HRP substrate) was then added and lastly, the reaction was stop using stop solution. The absorbance was read at 450 nm.

2.13. Statistical analysis

All statistical analyses were performed in Graph Pad Prism 9 (version 9.3.0, San Diego, CA). All results are presented as mean ± standard error. Data for fluid and food intake were analyzed by unpaired two-tailed student's t-test, for comparison between water and fructose at each time point. All other datasets were analyzed using one-way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc test for multiple comparison to determine significance differences among various groups. The groups considered for multiple comparisons were WSV *vs* WTV, WSV *vs* FSV, WSV *vs* FTV, WTV *vs* WTT4, FSV *vs* FST4, FTV *vs* FTT4. A value of p 0.05 was considered as statistically significant. Pearson correlation analysis was performed to assess association between two variables such as *de novo* lipogenesis, insulin signaling, inflammatory markers, lipid levels.

3. Results

3.1. Effects of thyroid hormone on glucose metabolism

We determined whether fructose consumption would influence the action of TBI on systemic glucose metabolism. Rats subjected to TBI (WTV) showed impaired glucose metabolism and slowly metabolized glucose in comparison to water-sham vehicle controls

(WSV) (p 0.05) (Fig. 1b and c). Moreover, the condition was farther worsened in fructose (FSV) (p 0.05) and fructose-TBI rats (FTV) (p 0.001) compared to water sham control (WSV). However, T4 treated fructose-TBI group (FTT4) showed higher glucose utilization as observed by AUC in comparison to fructose-TBI control, FTV (p 0.01) (Fig. 1b and c). It is well-known that rats exposed to fructose consumption compensate for their caloric gain by reducing their food intake. This is a known compensatory mechanism to maintain caloric homeostasis by co-regulating their fluid and food intake. There were no differences in the body weight across the different groups, and across time points (Supplementary Fig. S2a, 2b, 2c). It is reasonable to assume that the observed changes in glucose metabolism are due to overconsumption of fructose that was further aggravated by TBI pathogenesis.

3.2. Effects of T4 on insulin sensitivity

We evaluated the mRNA levels of insulin receptor (*Insr*), glucose transporter (*Glut 4*) and transthyretin (*Ttr*) in the liver to ascertain that the observed effects were not because of translocations from other organs. Our results showed that T4 treatment elevated *Insr* levels among TBI (WTT4) and fructose-TBI (FTT4) rats as compared to TBI (WTV) (p 0.05) and fructose-TBI (FTV) (p 0.05) (Fig. 2a). Further, we studied mRNA levels of glucose transporter 4 (*Glut 4*), involved in procuring insulin sensitivity. T4 administration elevated *Glut 4* mRNA in TBI (WTT4) and for fructose fed (FST4 or FTT4) rats as compared to TBI (WTV) (p 0.05), fructose (FSV) (p 0.01) and fructose-TBI (FTV) (p 0.01) groups, respectively (Fig. 2b). We also assessed the mRNA levels of the thyroid hormone transporter transthyretin (*Ttr*) and found that T4 treatment elevated *Ttr* level among fructose-fed TBI rats (FTT4 *vs* FTV) (p 0.001) (Fig. 2c). Our correlation analysis showed that mRNA levels of insulin receptor (*Insr*) changes in proportion to *Ttr* mRNA levels (Fig. 2d) (Pearson coefficient, r (33) = 0.4845, p = 0.0032). In addition, the mRNA levels of *Insr* increased in proportion to *Glut 4* mRNA in liver (Supplementary Fig. S1, (Pearson coefficient, r (36) = 0.3389, p = 0.0374).

We also studied the activational stage (phosphorylation) of key molecules responsible for regulating insulin signaling such as Akt serine/threonine kinase (AKT), glycogen synthase kinase-3 beta (GSK3P) and AMP-activated protein kinase (AMPK) in liver. While moderate reduction in pAkt levels were evident among fructose-fed group (FSV), a significant decrease in pGSK3β levels was observed when compared to water sham control (WSV) (p 0.05). However, T4 administration elevated pAKT (ser 473) among fructose-fed (FST4) rats when compared with fructose control group (FSV) (p 0.001) (Fig. 3a). Similarly, we found that T4 elevated phosphorylated levels of GSK3β (ser 9) in rats fed fructose (FST4) when compared with fructose control group (FSV; p 0.05) (Fig. 3b). Further, T4 treatment increased phosphorylation of AMPK among TBI (WTT4) and fructose-TBI (FTT4) groups when compared with regular water fed TBI (WTV) (p 0.01) and fructose-fed TBI (FTV) (p 0.01) groups (Fig. 3c). In addition, T4 administration increased phosphorylated levels of ACC, and downstream signaling molecule to AMPK, in rats fed fructose (FST4) and for fructose-TBI (FTT4), compared to fructose (FSV) (p 0.05) and fructose-TBI (FTV) (p 0.01) groups, respectively (Fig. 3d).

3.3. Effect of T4 on de novo lipogenesis, lipid peroxidation and lipid droplets formation

To determine the efficacy of T4 on *de novo* lipogenesis (DNL), we measured protein levels of key enzymes related to lipid metabolism such as fatty acid synthase (FAS) and acetyl-CoA synthetase (AceCS1). We found that TBI elevated levels of FAS, a rate limiting enzyme for *de novo* lipogenesis, and that T4 treatment counteracted this increase when compared with TBI (WTV) (p 0.05), fructose (FSV) (p 0.05) or fructose-TBI (FTV) (p 0.001) groups, respectively (Fig. 4a and b). Additionally, we assessed AceCS1 that catalyzes the synthesis of ACC, associated with fat accumulation and metabolism. Our results showed elevated levels of AceCS1 in fructose-TBI condition (FTV) compared to water sham (WSV) group (p 0.05). As shown Fig. 4a and c, T4 treatment counteracted the increasing effects of fructose among (FST4 *vs* FSV; p 0.05 and FTT4 *vs* FTV; p 0.001).

We assessed the levels of C16:0 (palmitic) and C18:2 (linoleic) fatty acids using gas chromatography. The relative levels of these fatty acids in the DNL index (C16:0/C18:2 ratio) provide an indication of the status of the plasma membrane and lipogenesis, such that the higher the DNL index (C16:0/C18:2), the greater the membrane damage and lipogenesis [26,27]. We observed an increase in the ratio in rats fed fructose (FSV) (p 0.0001) and among fructose-TBI group (FTV) (p 0.0001), as compared to water sham group (WSV). However, T4 treatment counteracted the increased DNL index compared to the respective injury controls, *i.e.*, WTV (p 0.05), FSV (p 0.05) and FTV (p 0.05) (Fig. 4d). Further, we determined the effect ofT4 on the end-product of lipid peroxidation 4-hydroxy-2-nonenal (4-HNE). As observed in Fig. 4g and h, fructose or TBI increased 4-HNE levels (FSV *vs* WSV, p 0.01 and FTV *vs* WTV, p 0.05) whereas T4 treatment counteracted the effect of fructose (FST4 *vs* FSV, p 0.05) and TBI (FTT4 *vs* FTV, p 0.01), respectively. The *de novo* lipogenesis index C16:0fC18:2 changed in inverse proportion to *Glut* 4 mRNA levels (Fig. 4e) (Pearson coefficient, r (35) = 0.5416, p = 0.0005). Additionally, the enzyme for lipogenesis, AceCS1 also changed in inverse proportion to *Glut* 4 mRNA levels (Fig. 4f) (Pearson coefficient, r (32) = 0.6220, p 0.0001) suggesting that increased *Glut* 4 gene expression could be a cause for reduced lipogenesis.

We performed Oil Red O staining in liver to provide a phenotypic assessment of the lipid/triglyceride accumulation. A tendency to form lipid droplets was observed to be higher in TBI rats (WTV) compared to water sham group (WSV). However, a significant increase in average number of lipid droplets was found with fructose exposure (FSV) (p 0.0001) and among fructose-TBI (FTV) group (p 0.0001), compared to water sham control (WSV). However, T4 treatment counteracted the effects of TBI (WTT4), fructose (FST4), and fructose-TBI (FTT4) on the average number (n) of lipid droplets as compared to their respective controls, TBI (WTV) (p 0.01), fructose (FSV) (p 0.0001) and fructose-TBI (FTV) (p 0.0001) (Fig. 5a and b). Moreover, the average area of the droplet was found to be moderately increased among TBI rats (WTV) while sharp increased among fructose-TBI (FTV) (p 0.001), compared to water sham control (WSV). However, T4 administration reduced the average lipid droplets among TBI rats (WTT4) and fructose-fed rats subjected to TBI (FTT4) when compared with TBI alone (WTV) (p 0.0001) or fructose-TBI (FTV) (p 0.001) groups (Fig. 5c).

3.4. Effects of T4 on Docosahexaenoic acid (DHA) and Arachidonic acid (AA) and pro-inflammatory markers

Since fatty acids are mainly metabolized in the liver, we assessed levels of the omega 3 fatty acid DHA and the omega-6 fatty acid arachidonic (AA) in liver in response to thyroid hormone administration employing gas chromatography. We observed a decline in DHA levels among fructose (FSV) (p 0.0001) and fructose-TBI (FTV) (p 0.0001) groups compared to water sham control (WSV). However, T4 treatment among TBI groups (FST4 or FTT4) counteracted the reducing effects of fructose (FSV) (p 0.0001) and fructose-TBI (FTV) (p 0.0001) on levels of DHA (Fig. 6a). In contrast to the effects of DHA, it has been reported that increased levels of AA induces inflammation and tissue damage [9,28,29]. We observed that T4 treatment normalized the levels of AA among TBI (WTT4), fructose (FST4) and fructose-TBI (FTT4) groups in comparison to TBI (WTV) (p 0.001), fructose (FSV) (p 0.01), and fructose-TBI (FTV) (p 0.0001) (Fig. 6b). Additionally, we found abnormal increase in ratio of AA:DHA among fructose (FSV) (p 0.0001) and fructose-TBI (FTV) (p 0.0001) groups when compared with water sham control (WSV). However, T4 administration reversed the disturbed AA:DHA ratio (Fig. 6c). A high AA:DHA ratio provides a general estimate of disrupted membrane integrityfrigidity and impaired lipid metabolism [30].

We assessed the levels of pro-inflammatory markers, *Tnfa* and *Mcp-1*, in the liver and plasma, and found increased levels of *Tnfa* in TBI group (WTV) and fructose group (FSV), as compared to water-sham control group (WSV) (p 0.05). However, as shown in Fig. 6d, T4 administration counteracted the *Tnfa* increase in response to fructose (FST4 *vs* FSV, p 0.01). Another inflammatory marker, *Mcp-1*, was also normalized with T4 administration in fructose (FST4) and fructose-TBI (FTT4) group in response to fructose (FSV) (p 0.05) and fructose-TBI (FTV) (p 0.05), respectively (Fig. 6e). Additionally, we observed that T4 administration reversed the increased levels of circulatory plasma Mcp-1 among TBI (WTT4) and fructose-TBI (FTT4) groups in comparison to TBI (WTV) (p 0.05) and fructose-TBI (p 0.05), respectively (Fig. 6f). Interestingly, correlation studies showed that the ratio AA/DHA changed in proportion to the mRNA levels of inflammatory marker, *Mcp-1* (Fig. 6g) (Pearson coefficient, r (35) = 0.4569, p = 0.0045).

3.5. Effects of T4 on insulin signaling and fat metabolism in primary hepatocytes

We examined the possibility that T4 could exert a direct effect on primary hepatocytes in culture during fructose-TBI condition. Consistent with the *in-vivo* effects of fructose-TBI on impaired lipid metabolism and triglyceride accumulation, we found an increase in FAS levels in fructose-TBI hepatocytes compared to sham (p 0.05) (Fig. 7a). However, exogenous T4 treatment (100 nM, 16 h) in fructose-TBI hepatocytes (F + TBI + T4) restored the FAS levels to normal as compared to fructose-TBI group (F + TBI) (p 0.05).

T4 treatment increased phosphorylation of AMPK at threonine 172 in primary hepatocytes isolated from mice exposed to TBI and fructose, as compared to fructose-TBI group (F + TBI) (p 0.01) (Fig. 7b). T4 treatment also increased the phosphorylation of AKT substrate AS160 in primary hepatocytes exposed to fructose and TBI (F + TBI + T4) *vs* fructose-TBI group (F + TBI) (p 0.001) (Fig. 7c). We did not observe any difference in the activation of

Akt and GSK3P after T4 treatment in injured hepatocytes (F + TBI + T4) *vs* fructose-TBI hepatocytes (F + TBI) (Fig. 7d and e). We used 2-NBDG fluorescence method to assess glucose uptake and found significant reduction in glucose uptake in the injury condition, *i.e.*, F + TBI in comparison to sham control group (p 0.01). However, a marked increase was observed in hepatocytes treated with T4 that originated from injured animals and exposed to fructose and TBI (F + TBI + T4), relative to controls (F + TBI) (p 0.0001) (Fig. 7f). Correlation analysis showed that the ratio pAMPKα/AMPK changes in proportion to relative levels of2-NBDG Suorescence (A.U.), and suggests that glucose uptake involves AMPK activation (Fig. 7g) (Pearson coefficient, $r(21) = 0.4630$, $P = 0.0261$). The above data emphasize the potential of T4 to support peripheral insulin sensitivity and lipid metabolism.

4. Discussion

Our results expand current views that TBI pathogenesis involves peripheral organs and tissues. Clinical reports indicate that TBI patients develop abnormalities in autonomic, neuroendocrine, and immune functions affecting metabolism of glucose, lipids, and overall metabolic regulation [31–33]. Our current results reveal that TBI has an impact on metabolic and inflammatory markers important for liver operation and that consumption of fructose contributes to the detrimental effects of TBI. The supply of the thyroid hormone T4 helped to improve several processes controlled by the liver. The overall evidence seems to indicate that the central role of liver on body physiology is part of a continuum operation with brain, in which, the liver works as a gate for the effects of injury and diet on brain pathologies.

4.1. Effects of TBI on the liver and counteractive action of T4

Several clinical reports indicate that TBI disrupts insulin signaling pathways and intensifies the progression of neurological disorders [34–36]. We previously reported that consumption of fructose disrupts hepatic glucose and fat metabolism and that these abnormalities are worsened after a TBI [7]. Our new results revealed that T4 administration normalized parameters of glucose metabolism, likely by sensitizing the insulin receptor. Liver is a crucial organ for maintaining glucose homeostasis and fructose metabolism such that patients suffering from liver cirrhosis exhibit impaired glucose metabolism/tolerance [37–40]. To evaluate the action of TBI and fructose, we assessed elements involved in the action of insulin signaling such as AKT, GSK, AMPK and how T4 affected the effects of TBI and fructose.

We show that fructose reduced the activation of AKT, and that T4 counteracts these effects by increasing phosphorylation of AKT, in addition to AMPK which regulates insulin signaling and play important roles during metabolic stress. AKT improves insulin sensitivity and glucose uptake by translocation of the glucose transporter GLUT 4 to the plasma membrane [41,42]. Our findings also show that fructose and TBI reduces the levels of *Glut 4* and AMPK. AMPK is an intracellular energy sensor and master regulator of cellular metabolism, and uses *Glut 4* translocation to improve glucose uptake [43]. AKT phosphorylates GSK3P and AMPK activates Acetyl CoA carboxylase (ACC), and they both influence *Glut 4* translocation to plasma membrane resulting in elevated glucose uptake and

insulin sensitivity [44,45]. The overall effects of T4 on AKT, AMPK, and *Glut 4* emphasize the therapeutic potential of T4 on promoting insulin sensitivity [46,47] and protecting against diabetes [48].

4.2. Action of TBI and T4 on liver lipogenesis

Lipids are integral components of brain and have a crucial action on cell signaling. Although most brain lipids are synthesized in the brain itself, subproducts of liver lipid synthesis are critical for maintaining brain function, particularly after TBI [49,50]. Our results show that TBI and for fructose affect lipogenic enzymes in the liver and suggest that T4 supports *de novo* lipogenesis (DNL), as evidenced by restoration of C16:0fC18:2 ratio in rats exposed to TBI and fructose. DNL is a complex and highly regulated metabolic process predominantly taking place in liver. It converts excess carbohydrates into specific fatty acids that are then esterified to store triacylglycerols as energy source. Hepatic fatty acid composition plays an important role in the activity of lipogenic pathways. The ratio of palmitic acid (C16:0) to linoleic acid (C18:2) confirms the DNL index [26,27] and our results show that TBI and for fructose increase DNL index, and that these effects were counteracted by T4 treatment.

AKT and AMPK activation in the liver has also been found to play important role in the regulation of DNL [51] and fat accumulation [52,53]. We presently found that T4 activates AMPK and ACC with potential inhibition of fatty acid/triglyceride synthesis and oxidation in liver. Interestingly, consistent with our observations, hyperlipidemic rodent model (characterized by lipid accumulation) exhibits reduced AMPK phosphorylation, hepatic lipid accumulation, and lipid toxicity which are consistent with non-alcoholic liver steatosis [54]. Further, consistent with the action of T4 on lipid metabolism, a recent study demonstrated increased accumulation of lipid droplets in mice associated with hypothyroidism fed with a high fat diet [55]. In addition, T4 administration also reduced the levels of the lipogenic enzymes FAS and AceCS1, supporting the possibility that T4 may act to reduce the accumulation of triglycerides and regulate lipid metabolism.

Lipid accumulation also recruits proinflammatory macrophages into the liver causing hepatic inflammation [56] which leads to lipid peroxidation and tissue damage [57–59]. Studies have shown that insulin resistance increases DNL leading to triglyceride accumulation by conversion of excessive carbohydrate into specific fatty acids [10]. Our current results show that TBI and for fructose increases the level of lipid peroxidation marker 4-HNE, while these changes were counteracted by T4 administration. 4-HNE is derived from poly-unsaturated omega-6 fatty acid [60] that negatively impacts cell integrity, cell metabolism and causes mutations [61,62]. The increased levels of 4-HNE have been linked to various diseases such as alcoholic liver disease [63], chronic obstructive pulmonary disease, emphysema, asthma [64,65], Alzheimer's and Parkinson's disease [66] and TBI [67]. Therefore, detoxification of 4-HNE is crucial for ameliorating disease pathogenesis [68–70]. Accordingly, our results showing that T4 reduces the levels of 4-HNE may be indicative of the restorative action of T4 on liver dysfunction after TBI and fructose. Importantly, we observed that thyroid hormone treatment reduced the elevation of the pro-inflammatory cytokines *Mcp-1* and *Tnfa* associated with fructose consumption and TBI.

Our results with primary cultured hepatocytes show that T4 directly acts on liver cells affected by fructose and TBI. The exogeneous administration of T4 to hepatocytes improved insulin signaling and reduced lipogenesis. These results are consistent with our *in-vivo* studies showing that T4 increases phosphorylation of the fuel sensing AMPK. Hence, the findings with primary hepatocytes help us to derive a mechanism by which insulin resistance can aggravate triglyceride accumulation involved in liver disorders such as steatosis and NAFLD pathogenesis [71–73].

4.3. Action of TBI and T4 on omega-3 fatty acids

Omega-3 fatty acids are metabolized in the liver, and we found that TBI and fructose reduced the level of DHA in the liver. This finding emphasizes the role of the liver on the control of DHA function during the TBI pathology [74], and suggests that liver dysfunction can reduce efficacy of DHA treatment. It is also noteworthy that thyroid hormone treatment counteracted the reduction in DHA levels. DHA is an essential structural component of plasma membranes, and the lack of DHA compromises plasma membrane integrity [75] and insulin signaling [76] in brain [9], which can be prevented by exogenous DHA [9]. Our results show that T4 reduced the ratio of n-6:n-3 fatty acids and increases levels of DHA which can improve membrane fluidity and function. The outcomes the current study are in agreement with the results of previous research showing that an omega-3 rich diet potentiates thyroid hormone signaling in the liver and neutralizes *de novo* lipogenesis, fatty acid oxidation and triglyceride accumulation [77].

In conclusion, our study shows that TBI disrupts molecular systems in the liver which are necessary for the maintenance of metabolic operations, and that these effects are aggravated by the consumption of fructose. T4 administration mitigated the actions of fructose and TBI on liver metabolism. T4 was found to support fat/lipid metabolism by reducing *de novo* lipogenesis, lipogenic enzymes (FAS, ACC, AceCS1) and lipid peroxidation, leading to reduced triglyceride accumulation and lipid droplets formation. T4 was found to augment insulin sensitivity by increasing insulin signaling and glucose tolerance (Fig. 8). In addition, T4 showed to restore the metabolism of DHA in the liver disrupted by TBI and fructose. The overall evidence seems to indicate that the liver works as a gate for the regulation of the effects of brain injury and foods on brain pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

This work was funded by National Institutes of Health (NIH) grants; R01-NS111378, RO1-NS117148 and RO1-NS116383.

Data availability

Data will be made available on request.

Abbreviations:

TBI	Traumatic Brain Injury
FPI	Fluid percussion injury
DHA	Docosahexaenoic acid
T4	Thyroid hormone or L-thyroxine sodium salt pentahydrate
AA	Arachidonic acid
C16:0	Palmitic fatty acid
C18:2	Linoleic fatty acid
ACC	Acetyl CoA carboxylase
AceCS1	Acetyl-CoA synthetase
FAS	Fatty acid synthase
TNFα	Tumor necrosis factor alpha
MCP1	Monocyte chemoattractant protein-1
AKT	Akt serine/threonine kinase
AMPK	AMP-activated protein kinase
GSK3β	Glycogen synthase kinase-3 beta
AS160	Akt substrate160
Insr	Insulin receptor
DNL	*De novo* lipogenesis
Ttr	Transthyretin
GLUT	glucose transporter
2-NBDG	(2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose)
GC	Gas chromatography
4-HNE	4-hydroxy-2-nonenal.

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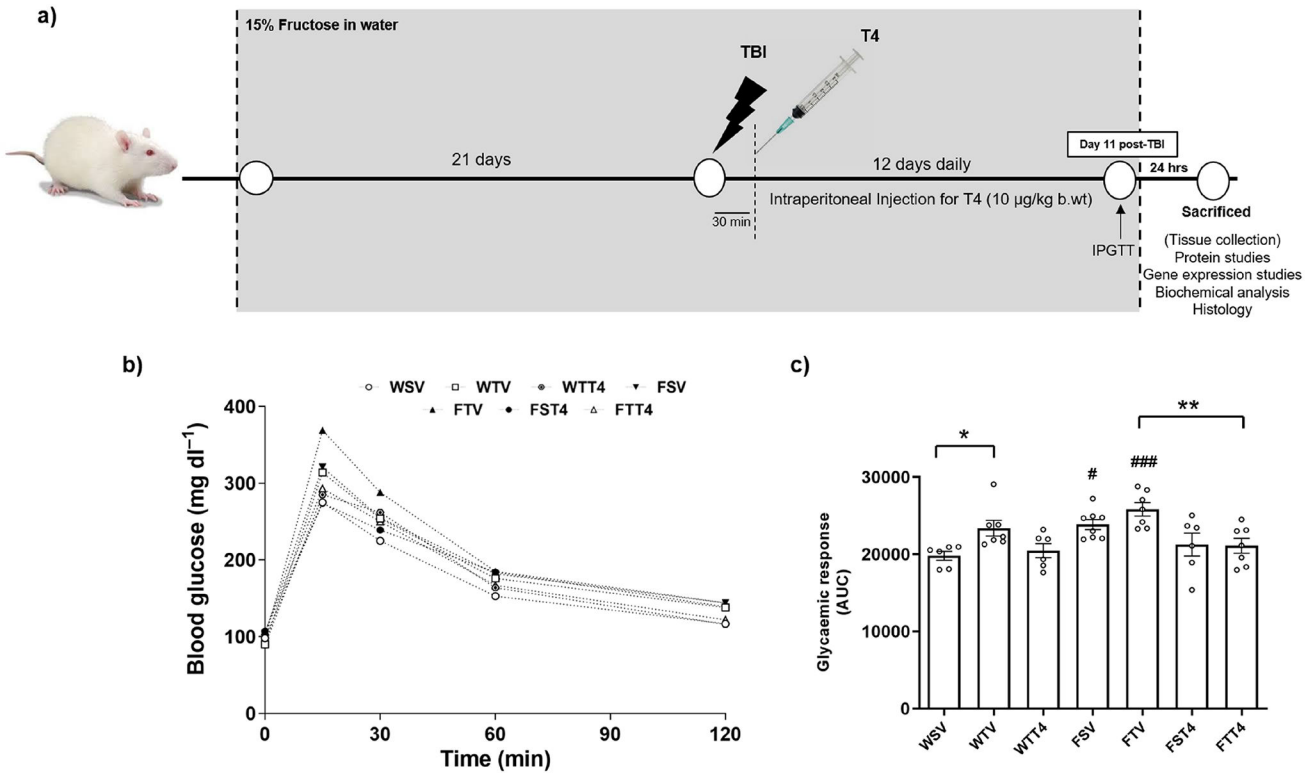

Fig. 1.

a) Experimental Design; b) Glucose Tolerance test (GTT); c) Area under the curve (AUC). Data was analyzed using one-way ANOVA, F (6, 40) = 5.27, $P = 0.0004$ followed by Holm-Sidak *post hoc* test for multiple comparison. Data are expressed as mean ± SEM, n = 6–7 rats/group. *p 0.05, **p 0.01, #p 0.05 (WSV *vs* FSV/FTV), $^{###}p$ 0.001 (WSV *vs* FSV/FTV).

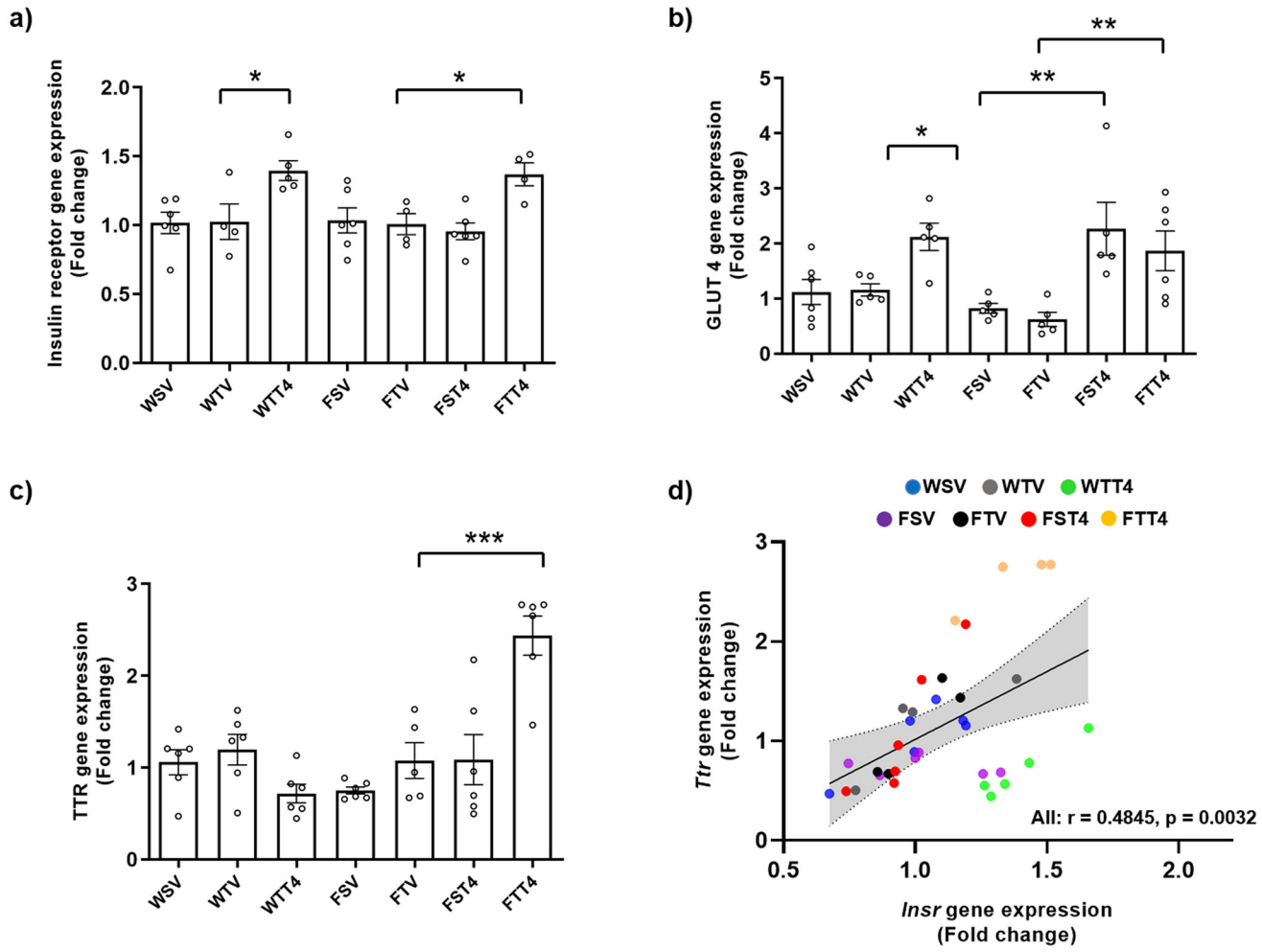

Fig. 2.

T4 augments insulin sensitivity in liver: mRNA expression of a) Insulin receptor (*Insr*) (one way ANOVA, F (6, 28) = 4.60, $P = 0.0023$), $n = 4–6$ rats/group; b) *Glut 4* (one way ANOVA, F (6, 30) = 5.43, $P = 0.0007$), $n = 5–6$ rats/group; c) *Ttr* (one way ANOVA, F (6, 34) = 11.21, *P* 0.0001), n = 5–6 rats/group; d) Correlation between *Ttr* (fold change) and *Insr* (fold change). Data are expressed as mean ± SEM. *p 0.05, **p 0.01, ****p* 0.001. Data was analyzed one-way ANOVA followed by Holm-Sidak *post hoc* test.

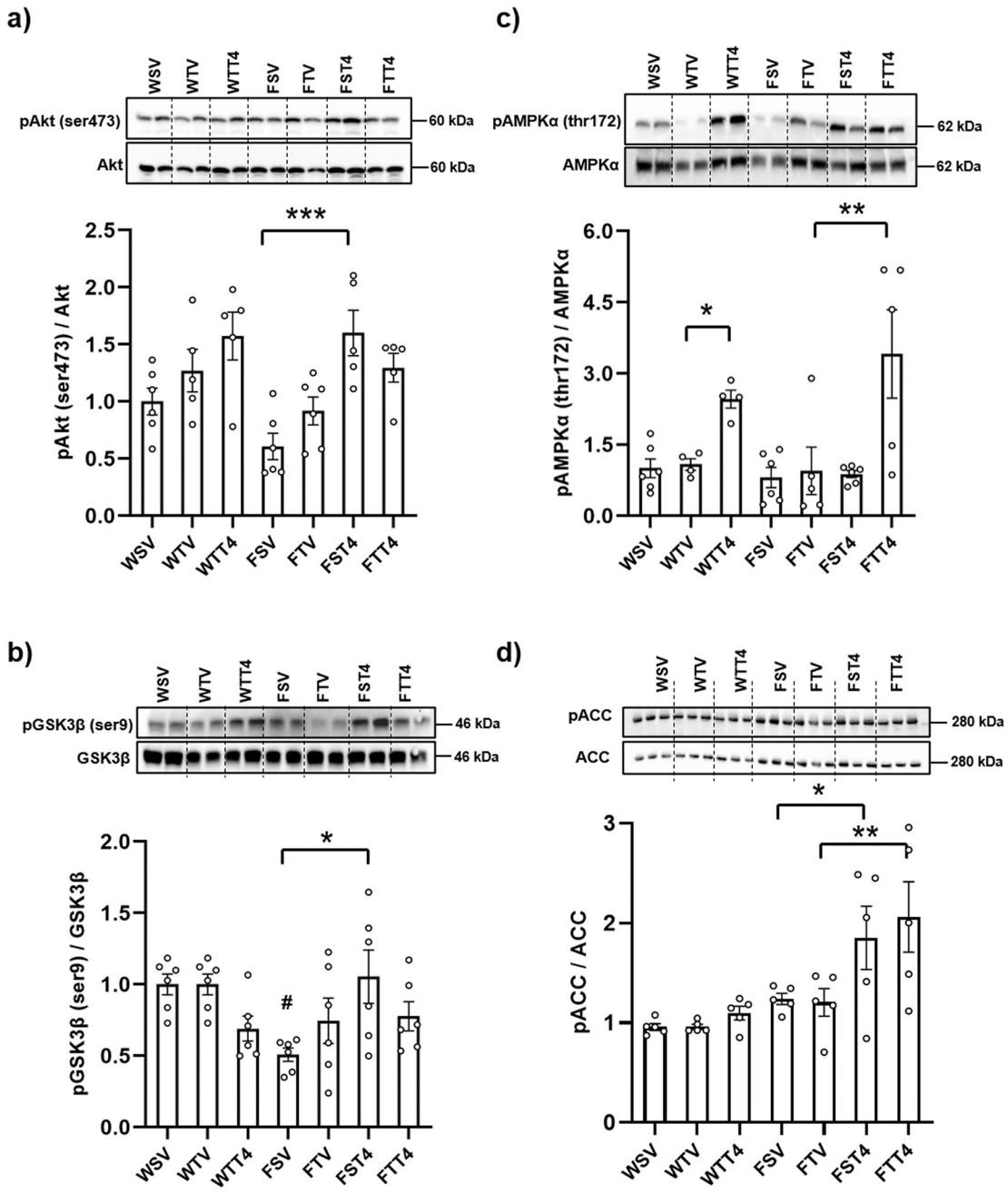

Fig. 3.

T4 augments insulin sensitivity in liver: Representative western blots and densitometric analysis of insulin signaling molecules compared to their respective total protein levels; a) pAKT (ser 473) (one way ANOVA, F (6, 31) = 5.66, $P = 0.0005$) $n = 5–6$ rats/group; b) pGSK (ser 9) (one way ANOVA, F (6, 35) = 3.09, $P = 0.0156$), n = 6 rats/group; c) pAMPK (thr 172) (one way ANOVA, F (6, 29) = 5.77, P = 0.0005), n = 4–6 rats/group; d) pACC (one way ANOVA, F (6, 28) = 5.32, $P = 0.0009$) n = 5–6 rats/group. Data are expressed as mean ± SEM. *p 0.05, **p 0.01, ***p 0.001. Data were analyzed using one-way ANOVA followed by Holm-Sidak *post hoc* test.

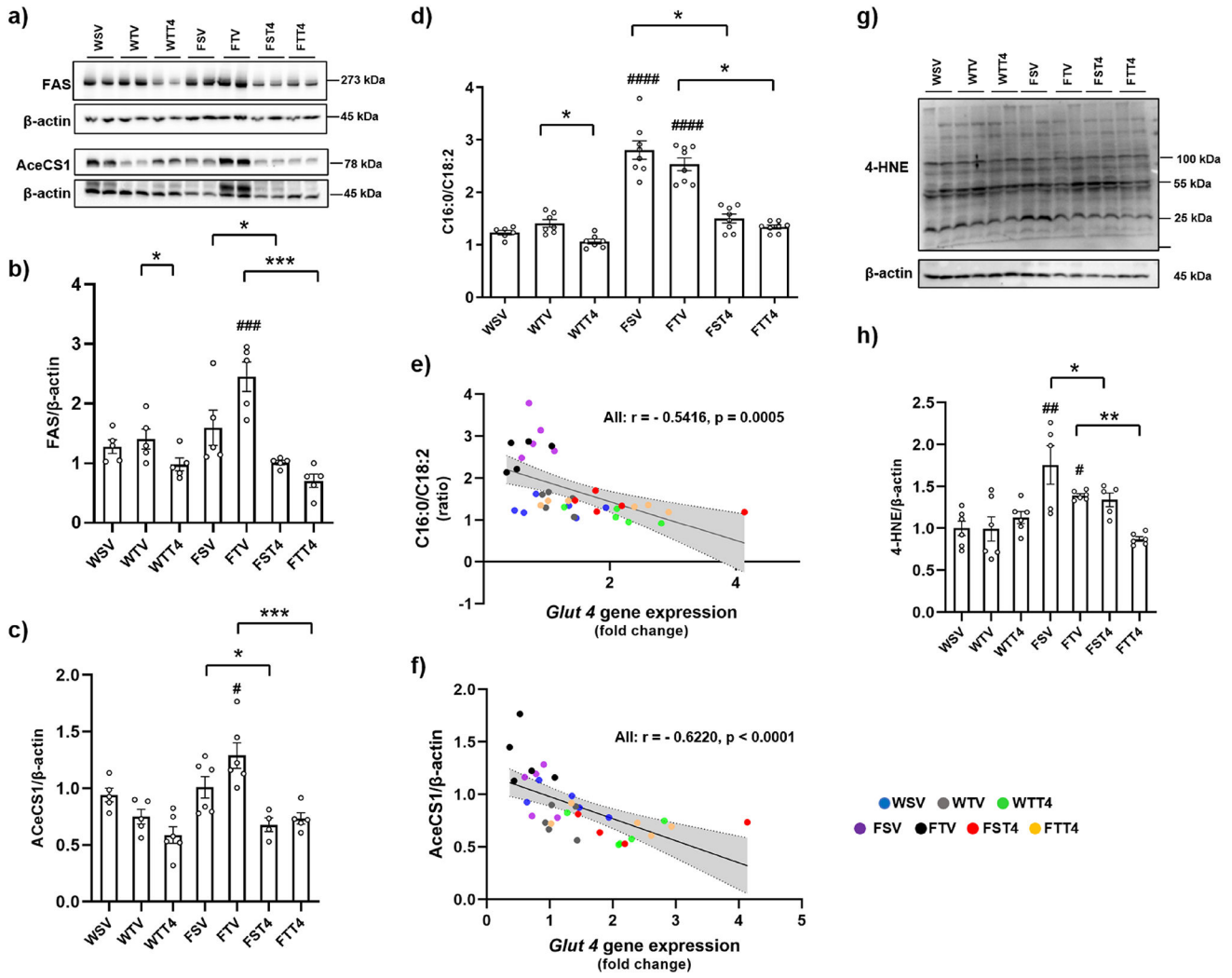

Fig. 4.

T4 suppresses *de novo* lipogenesis in liver and reduces lipid accumulation in liver: a) Representative western blots for FAS, AceCS1 and β-actin; b) Densitometric analysis for FAS compared to β-actin (one way ANOVA, F (6, 28) = 10.85, *P* 0.0001), *n* = 5–6 rats/group; c) Densitometric analysis for AceCS1 compared to β-actin (one way ANOVA, F (6, 30) = 9.42, *P* 0.0001), n = 4–6 rats/group; d) Ratio of C16:0/C18:2 (one way ANOVA, F (6, 49) = 48.52, *P* 0.0001), *n* = 7–8 rats/group; e) Correlation between C16:0/C18:2 and *Glut* 4 mRNA; f) Correlation between AceCS1/β-actin and *Glut* 4 mRNA; g) Representative western blots for 4-HNE; h) Densitometric analysis for 4-HNE compared to β-actin (one way ANOVA, F (6, 33) = 7.41, *P* 0.0001), *n* = 5–6 rats/group. Results are expressed as mean ± SEM. **p* 0.05, ***p* 0.01, ****p* 0.001, *****p* 0.0001, $^{\#}$p 0.05 (WSV *vs* FSV/FTV), $^{\#\#}$p 0.01 (WSV *vs* FSV/FTV), $^{\#\#\#}$*p* 0.001 (WSV *vs* FSV/FTV), $^{\#\#\#\#}$*p* 0.0001 (WSV *vs* FSV/FTV). Data were analyzed using one-way ANOVA followed by Holm-Sidak *post hoc* test.

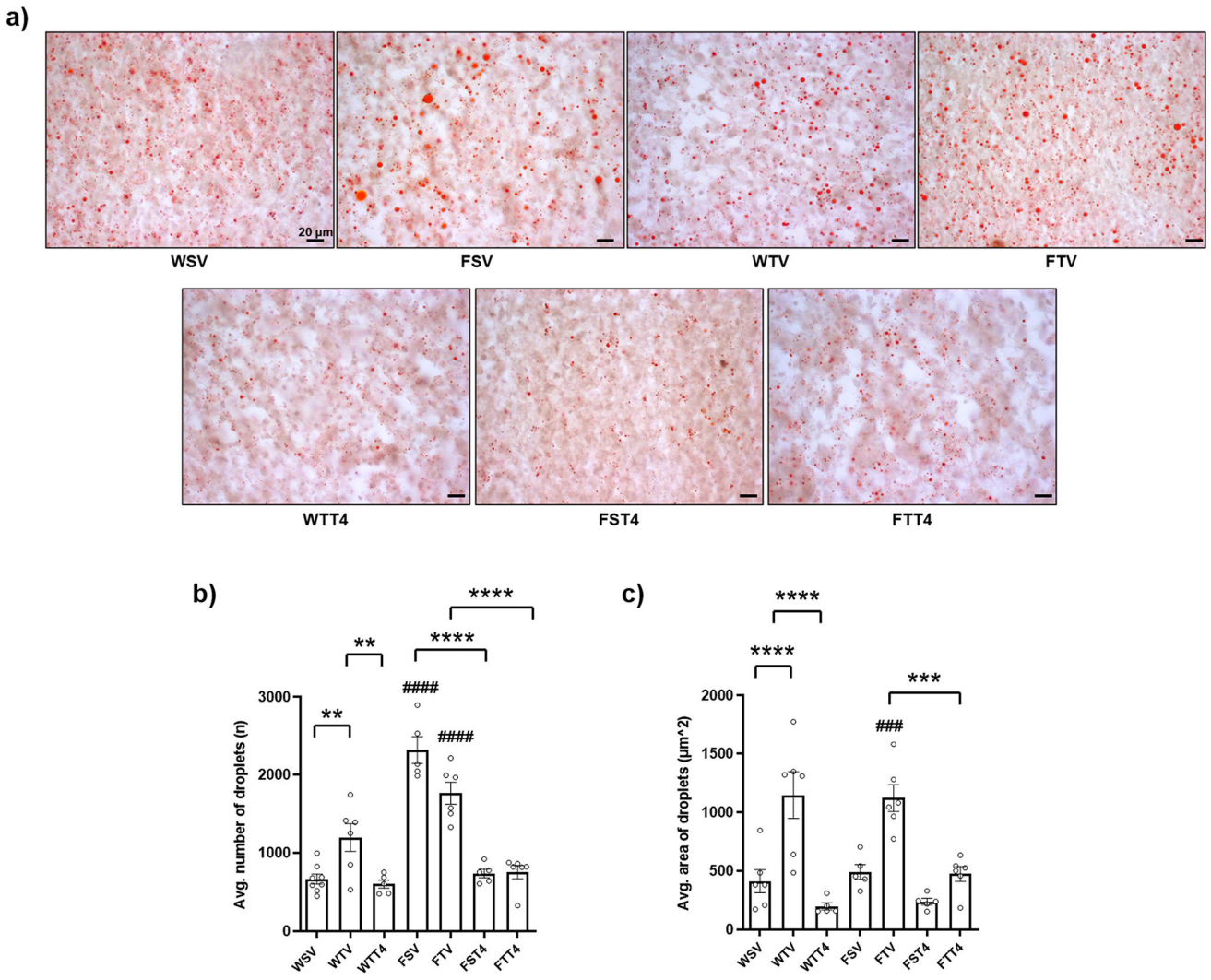

Fig. 5.

T4 reduces lipid droplets formation: a) oil droplets staining (ANOVA, P 0.0001); b) Avg. number of droplets (n) (one way ANOVA, F (6, 34) = 29.82, P 0.0001); c) Avg. size of droplets µm^2) (one way ANOVA, F (6, 32) = 13.32, P 0.0001). Data are expressed as mean ± SEM, n = 5–6 rats/group. **p* 0.05, ***p* 0.01, ***p 0.001, ****p 0.0001, $^{\#\#\#}$p 0.0001 (WSV *vs* FSV/FTV). Data were analyzed using one-way ANOVA followed by 0.001 (WSV *vs* FSV/FTV), $^{\#\#\#\#}$p Holm-Sidak *post hoc* test.

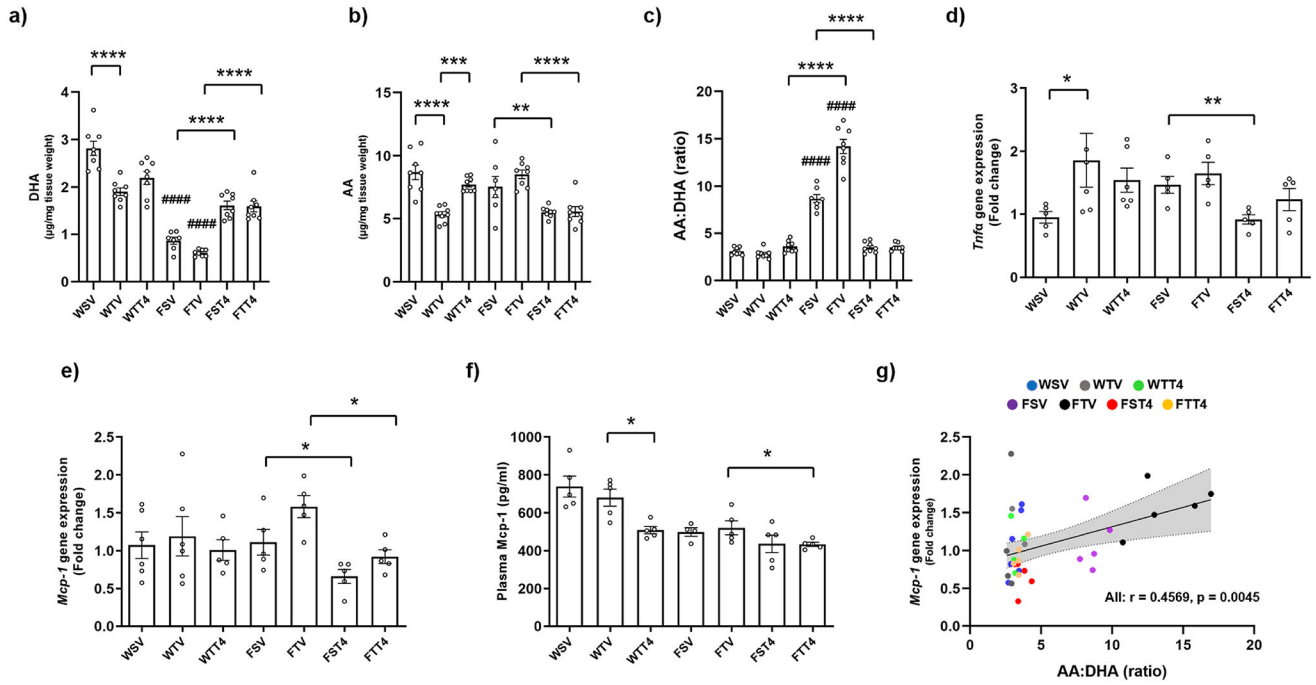

Fig. 6.

T4 modulates DHA and AA fatty acids, improves membrane integrity and reduces inflammation in liver: Gas Chromatography analysis for a) DHA (one way ANOVA, F (6, 48) = 52.10, *P* 0.0001), n = 7–8 rats/group; b) AA (one way ANOVA, F (6, 48) = 12.12, P 0.0001), n = 7–8 rats/group; c) ratio AA:DHA (one way ANOVA, F (6, 48) = 146.7, P 0.0001), n = 7–8 rats/group; d) mRNA expression of *Tnfα* (one way ANOVA, F (6, 30) = 2.39, *P* = 0.0071), n = 5–6 rats/group; e) mRNA expression of *Mcp-1* (one way ANOVA, F (6, 30) = 2.54, *P* = 0.0414), n = 5–6 rats/group; f) ELISA for plasma concentration (pg/ml) of Mcp-1 (one way ANOVA, F (6, 27) = 10.19, P 0.0001), *n* = 4–5 rats/group; g) Correlation between *Mcp-1* gene expression and AA:DHA ratio. Data are expressed as mean ± SEM, *n* = 6–7 rats/group. **p* 0.05, ***p* 0.01, ****p* 0.001, ****p 0.0001, ####p 0.0001 (WSV *vs* FSV/FTV). Data were analyzed using one-way ANOVA followed by Holm-Sidak *post hoc* test.

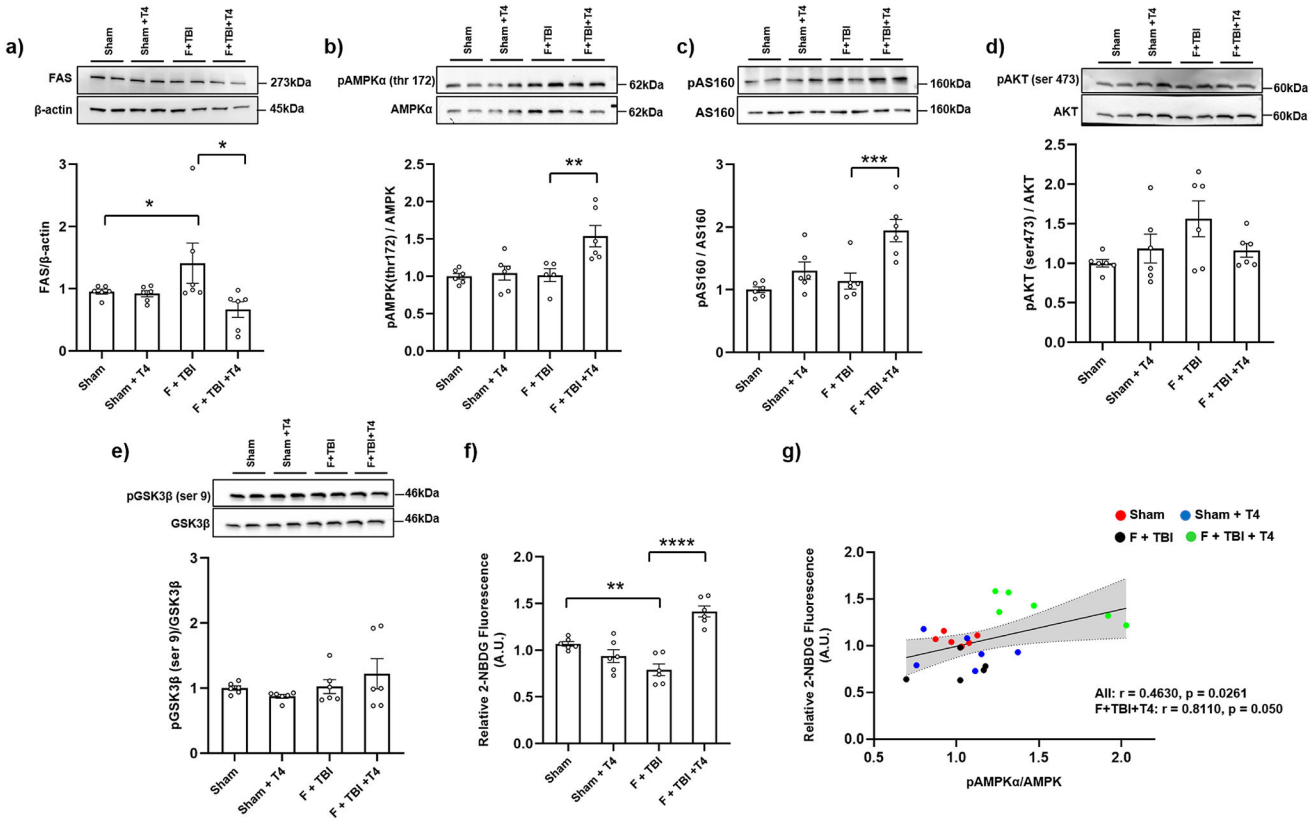

Fig. 7.

Direct effect of T4 on insulin signaling and fat metabolism in primary hepatocytes: Representative western blots and densitometry compared to total/β-actin; a) FAS (one way ANOVA, F (3,20) = 3.09, $P = 0.0502$); b) pAMPK (thr172) (one way ANOVA, F (3, 19) = 7.05, $P = 0.0022$); c) pAS160 (one way ANOVA, F (3, 20) = 10.04, $P = 0.0003$); d) pAKT (ser 473) (one way ANOVA, F (3, 20) = 2.38, $P = 0.0996$); e) pGSK3β (ser 9) (one way ANOVA, F (3, 20) = 1.22, $P = 0.3285$); f) Relative 2-NBDG Suoresence (A.U.) (one way ANOVA, F (3, 20) = 22.13, P 0.0001); g) Correlation between levels of pAMPKα (thr172)/AMPK and relative 2-NBDG fluorescence (A.U.). Three independent experiments were performed in replicates. Data are expressed as mean ± SEM. *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001. Data were analyzed using one-way ANOVA followed by Holm-Sidak *post hoc* test.

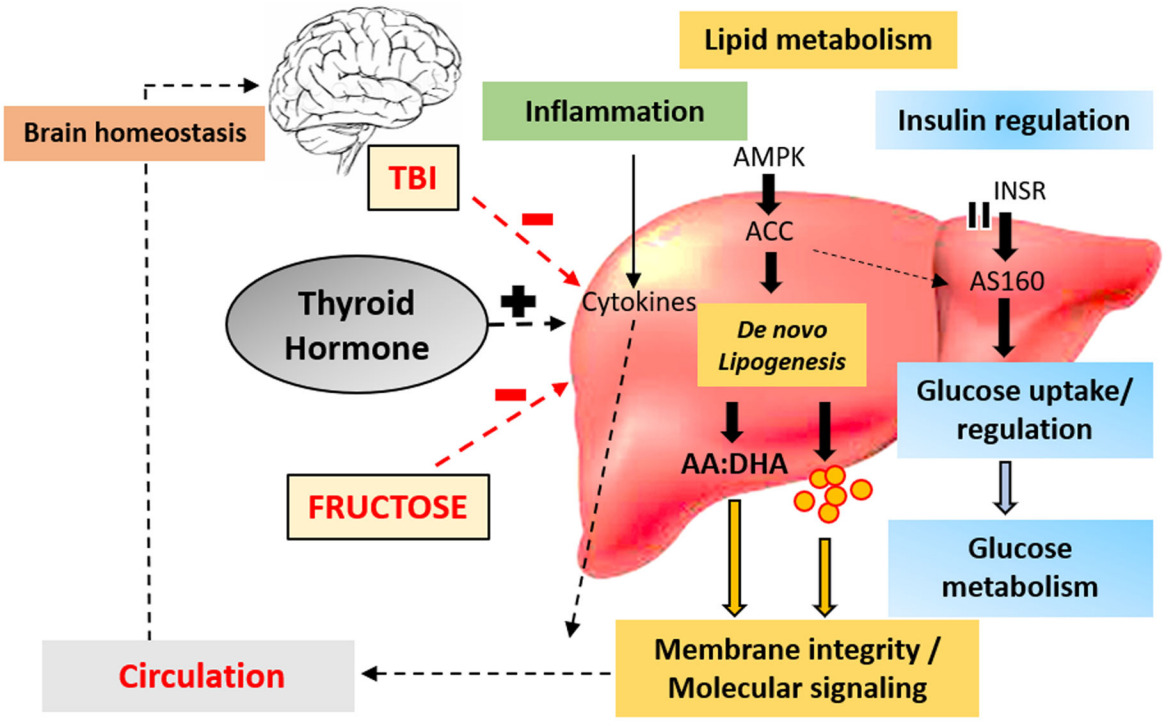

Fig. 8.

Graphical Abstract: Our study shows that TBI disrupts molecular systems in the liver which are necessary for the maintenance of metabolic operations and brain function, and that these effects are aggravated by the consumption of fructose. T4 supports fat/lipid metabolism by reducing *de novo* lipogenesis, lipogenic enzyme ACC, and lipid peroxidation, leading to reduced triglyceride accumulation and lipid droplets formation, in which AMPK plays a role. T4 also potentiates insulin sensitivity after TBI and for high fructose intake by acting on Akt substrate AS160 leading to increased glucose uptake and metabolism. T4 also preserves the metabolism of DHA and normalizes the ratio of arachidonic acid (AA):DHA that provides an index of healthy plasma membrane disrupted by TBI and fructose. The overall evidence seems to indicate that the liver works as a gate for the regulation of the effects of brain injury and foods on brain pathologies.

Table 1

Experimental groups.

Group No.	Treatment	Group Representation
1	Water − Sham + Vehicle	WSV
2	Water − TBI + Vehicle	WTV
3	Water − TBI + T4	WTT4
4	Fructose − Sham + Vehicle	FSV
5	Fructose − TBI + Vehicle	FTV
6	Fructose − Sham + T4	FST4
7	Fructose − TBI + T4	FTT4

Table 2

List of Antibodies.

Antibody	Company	Catalog No.	Molecular Weight (kDa)
pAMPKα (thr 172)	Cell signaling	2535	62
AMPKα	Cell signaling	2603	62
pACC	Cell signaling	3661	280
ACC	Cell signaling	3662	280
pAKT (ser 473)	Cell signaling	4060	60
AKT	Cell signaling	4691	60
pGSK3β (ser 9)	Cell signaling	9336	46
GSK3β	Cell signaling	12456	46
FAS	Cell signaling	3180	273
AceCS1	Cell signaling	3658	78
pAS160	Cell signaling	8619	160
AS160	Cell signaling	2670	160
4-HNE	Millipore	AB5605	multiple
β -actin	Santa Cruz	sc-47778	45

Table 3

List of rat primer sequences used in Quantitative real time PCR.

Gene	Accession number	Forward Primer (5′- 3′)	Reverse Primer (5′- 3′)
Glut 4	NM_012751.1	GTTGGTCTCGGTGCTCTTAG	GGCCACGATGGACACATAAC
Insr	NM 017071.2	TTCGAGGAGAGACCTTGGAA	TCGTGAGGTTGTGCTTGTTC
Ttr	NM_012681.2	ATGGTCAAAGTCCTGGATGC	GCCAAGAGCCTTCCAGTATG
Tnfα	NM_012675.3	TACTCCCAGGTTCTCTTCAAGG	GGAGGCTGACTTTCTCCTGGTA
Mcp-1	NM 031530.1	TAGCATCCACGTGCTGTCTC	CAGCCGACTCATTGGGATCA
Gapdh	NM_017008.4	GGGCTCTCTGCTCCTCCCTGT	ACGGCCAAATCCGTTCACACC

Biochim Biophys Acta Mol Basis Dis. Author manuscript; available in PMC 2023 October 26.