Sugar and brain disfunction: persistence of molecular alterations induced by a high-fructose diet in a juvenile rodent model

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Abstract

Introduction and Aims

Young age is often characterized by high consumption of processed foods, sodas, and fruit juices rich in high-fuctose corn syrup (HFCS), which is corn syrup. In recent years it has emerged that this compound, widely used in industry as a sweetener, induces not onlyoverweight, dyslipidemia, liver damage and metabolic syndrome in general, but can also cause alterations in brain function. The objectives of this PhD project were to (a) clarify the cerebral effects deriving from the consumption of fructose at a young age, a critical phase for brain development, and (b) study whether the cerebral alterations induced by an excess of sugars are abolished or persist after the elimination of the sugar and the switch to a balanced diet.

The study was conducted both on the hippocampus and frontal cortex, two areas of the brain involved in learning and memory, particularly susceptible to nutritional insults, and on the hypothalamus, an important district for the regulation of energy balance.

Results and Conclusions

In the first section of the thesis, the research data, obtained by feeding young rats with a short-term fructose-enriched diet, showed that this sugar induces mitochondrial dysfunction and oxidative stress in the hippocampus, associated with an increased concentration of inflammatory markers and decreased markers of synaptic function. Most of these changes in the hippocampus, with the exception of the increase in nitrotyrosine and the inflammatory marker haptoglobin, returned to values comparable to physiological conditions when the animals were returned to a balanced control diet. The results obtained were published in a scientific article (Mazzoli et al, Antioxidants 2021).

A further aim of this research project was to investigate the consequences induced by fructose-rich diet inthe frontal cortex. In fact, since the maturation of this district continues until late adolescence, it represents an interesting area to clarify the effects of added sugars in a critical period of maturation of the nervous system. The research data, obtained with the same experimental paradigm mentioned above, showed that fructose intake induces inflammation and oxidative stress in the frontal cortex, as well as changes both in brain-derived neurotrophic factor (BDNF) and in downstream markers of its signaling, such as cAMP response element binding protein (CREB) and in synaptic proteins. In addition, fructose-induced changes in the levels of key neurotransmitters, namely acetylcholine, dopamine and glutamate, as well as increased formation of glycation end products Nε-carboxymethyllysine (CML) and Nε-carboxyetyllisine (CEL) were measured. Importantly, many of these alterations (BDNF, CML, CEL, acetylcholinesterase activity, dysregulation of neurotransmitter

levels) persisted after switching to the control diet. The obtained results were very recently published in a scientific article (Spagnuolo et al, Mol Neurobiol 2022).

Finally, a further step of the research project was to evaluate the effect of this dietary treatment on the hypothalamus, a cerebral district involved in maintaining energy homeostasis. The results, recently published in a scientific article (D'Ambrosio et al, Nutrients 2023), indicate that a short-term fructose-rich diet strongly influences the mitochondrial and cytoskeletal compartments, as well as the level of specific markers of brain function. These alterations were restored after the removal of fructose from the diet. Collectively, these results indicate the harmful effects of excessive fructose consumption at a young age, and highlight that adolescence represents a critical phase, in which extreme care must be taken to limit excessive consumption of sweet foods that can affect brain physiology not only during their intake but also in the long term.

Riassunto

Introduzione e Obiettivi

La giovane età è spesso caratterizzata da un elevato consumo di alimenti processati, bibite gassate e succhi di frutta ricchi di *high-fuctose corn syrup* (HFCS), ovvero lo sciroppo di mais. Negli ultimi anni è emerso che questo composto, ampiamente utilizzato come dolcificante nell'industria, oltre a indurre sovrappeso, dislipidemia, danni al fegato e sindrome metabolica in generale, può anche provocare alterazioni della funzionalità cerebrale. Gli obiettivi di questo progetto di dottorato sono stati quelli di (a) chiarire gli effetti cerebrali derivanti dal consumo di fruttosio in età giovanile, una fase critica per lo sviluppo cerebrale, e (b) studiare se le alterazioni cerebrali indotte da un eccesso di zuccheri sono abolite o permangono dopo l'eliminazione dello zucchero ed il ritorno ad una dieta equilibrata.

Lo studio è stato condotto sia su ippocampo e corteccia frontale, due aree del cervello implicate nell'apprendimento e nella memoria, particolarmente suscettibili agli insulti nutrizionali, sia su ipotalamo, un distretto importante per la regolazione del bilancio energetico.

Risultati e conclusioni

Nella prima sezione della tesi, i dati della ricerca, ottenuti alimentando ratti giovani con una dieta arricchita in fruttosio di breve durata, hanno evidenziato che questo zucchero induce nell'ippocampo, disfunzione mitocondriale e stress ossidativo, associati a un'aumentata concentrazione di marcatori infiammatori e alla diminuzione di marcatori di funzionalità sinaptica. La gran parte di questi cambiamenti trovati nell'ippocampo, ad eccezione dell'aumento di nitrotirosine e del *marker* di infiammazione aptoglobina, ritorna a valori paragonabili alle condizioni fisiologiche riportando gli

animali ad una dieta di controllo equilibrata. I risultati ottenuti sono stati pubblicati su un articolo scientifico (Mazzoli et al, Antioxidants 2021).

Un obiettivo successivo di questo progetto di ricerca è stato quello di approfondire ulteriormente le conseguenze indotte da una dieta ricca in fruttosio sulla corteccia frontale. Infatti, poiché la maturazione di questo distretto continua fino alla tarda adolescenza, si tratta di un'area particolarmente interessante per chiarire gli effetti degli zuccheri aggiunti in un periodo critico della maturazione del sistema nervoso. I dati della ricerca, ottenuti con lo stesso paradigma sperimentale sopra menzionato, hanno mostrato che l'assunzione di fruttosio induce, nella corteccia frontale, infiammazione e stress ossidativo, oltre che cambiamenti sia nel *brain-derived neurotrophic factor* (BDNF) che in marcatori a valle del suo signaling e in proteine sinaptiche. Inoltre, sono stati misurati cambiamenti indotti dal fruttosio nei livelli di neurotrasmettitori chiave, ovvero acetilcolina, dopamina e glutammato, nonché aumento della formazione di prodotti finali di glicazione Nε-carbossimetillisina (CML) e Nε-carbossietillisina (CEL). È importante sottolineare che molte di queste alterazioni (BDNF, CML, CEL, attività dell'acetilcolinesterasi, disregolazione dei livelli di neurotrasmettitori) persistevano dopo il passaggio alla dieta di controllo. I risultati ottenuti sono stati recentemente pubblicati su un articolo scientifico (Spagnuolo et al, Mol Neurobiol 2022).

Infine, un ulteriore passo della sperimentazione è stato quello di valutare l'effetto di questo trattamento dietetico a livello ipotalamico, un distretto cerebrale implicato nel mantenimento dell'omeostasi energetica.I risultati, pubblicati di recente su un articolo scientifico (D'Ambrosio et al, Nutrients 2023), indicano che una dieta ricca in fruttosio di breve durata influenza significativamente i compartimenti mitocondriali e citoscheletrici, nonché il livello di specifici marcatori di funzionalità cerebrale; queste alterazioni vengono ripristinate dopo la rimozione del fruttosio dalla dieta. Complessivamente, questi risultati indicano gli effetti dannosi del consumo eccessivo di fruttosio in giovane età, ed evidenziano che l'adolescenza rappresenta una fase critica, in cui occorre prestare estrema attenzione nel limitare un consumo eccessivo di cibi ricchi di zuccheri aggiunti che possono avere non solo effetti deleteri sulla fisiologia cerebrale nel corso della loro assunzione ma anche effetti prolungati nonostante il ritorno a diete bilanciate povere di zuccheri.

Chapter 1

Introduction and research plans

Introduction

Changes in dietary lifestyle, such as the dramatic abuse of processed foods (bakeries, snacks, breakfast cereals) and bottled fruit juices rich in HFCS, particularly among young people, might have deleterious impacts on the body as well as on brain health by disrupting neuronal metabolism and function (Spagnuolo et al.,2020). Since high doses of fructose (≥1 g/kg) overwhelm intestinal fructose absorption and clearance, resulting in fructose reaching in the systemic circulation (Jang et al.,2018), a critical issue is determining how the intake of fructose can induce changes in brain structure and function.This PhD work elucidates, for the first time, themes of primary importance that have not yet been deeply investigated: (i) the effect of short-term fructose intake on hippocampus and frontal cortex function in the juvenile phase, which are critical for brain development and function, and ii) the putative reversibility of alterations induced by this sugar by switching to a control diet.
Furthermore, due to complexity and multiplicity of hypothalamic functions, a further objective of this PhD work was to highlight, in the hypothalamus, the early molecular alterations triggered by a sugar-rich diet throughout adolescence, and to verify their persistence until young adulthood phase.

Fructose metabolism

Fructose is a sugar naturally-occurring in fruits and vegetables, so it might not be judged to be as harmful as other types of sugars. The other common dietary sugars are glucose and sucrose, a disaccharide made of one fructose and one glucose molecule. Fructose also appears in its free form as high fructose corn syrup (HFCS), which has progressively replaced other addes sugars and became increasingly prevalent in the diet since the early 1970s (Smith et al., 1988). HFCS is used in sweetened beverages and processed foods and now comprises 40% of all caloric sweeteners (Bray et al., 2004; Fields et al., 2004; Goran et al., 2013b; Stanhope et al., 2015) because it is cheaper to produce, tastes sweeter, and increases the palatability of drinks, baked goods, or processed foods. The use of HFCS has led to an increase in daily fructose intake, where fructose may comprise approximately 15–21% of daily calories (Azais-Braesco et al., 2017). The World Health Organization recommends that added sugars should be limited to just 10% of our diet (World Health Organization, 2015) and yet fructose intake in young people may exceed 15% of total caloric intake (Vos et al., 2008). Excessive fructose intake can be problematic because unlike glucose, which is used by all cells as an energy source, fructose is broken down primarily by the intestine and liver to stimulate lipogenesis (Jang et al., 2018). At small amounts, fructose can be absorbed and broken down entirely by the small intestine, and fructose absorption can lead to increases in plasma triglycerides (Steenson et al., 2020; Theytaz et al., 2014). However, large amounts of fructose can overload the intestine and spillover

into the liver where it is a substrate for fatty acid synthesis and contributes to the accumulation of fatty liver and adiposity (Faeh et al., 2005; Schwarz et al., 2015; Stanhope et al., 2009; Taskinen et al., 2019). Furthermore, while glucose is broken down to generate substrates for energy production via glycolysis, the entry of fructose-derived intermediates into the glycolytic pathway is not regulated by negative feedback, so there is an increased availability of substrates for metabolic pathways like lipogenesis (Samuel et al., 2011). The primary metabolites and by-products of fructolysis in liver are glucose, lactate, free fatty acids, very low-density lipoproteins, uric acid and methylglyoxal, which can impair the function of extrahepatic tissues and organs. In addition to rapid fructolysis in liver, high fructose causes an ATP depletion that triggers inflammatory response and oxidative stress, thereby disturbing functions of local tissues and organs. Subsequently, inflammatory cytokine, adiponectin, leptin, and endotoxin are produced and become indirect dangerous factors (Zhang et al., 2017). Both clinical investigations and animal studies have shown that fructose and its metabolites directly and/or indirectly cause insulin resistance, chronic inflammation, onset of dyslipidemia, autophagy, and appetite disturbances for food intake (Zhang et al., 2017; Schulze et al., 2004; Livesey et al., 2008; Alwahsh et al., 2017). Evidence from epidemiological studies has suggested that fructose is a potential contributor to the worldwide increase in overweight and metabolic diseases (Bray et al., 2004; Bray et al., 2010; Stenhope et al., 2012).

Fructose can enter the brain to regulate feeding neural circuitry and food intake

Emerging evidence in recent years strongly suggests that fructose can enter the brain compartment, be metabolised there, and exert its direct action on CNS cells. In fact, fructose not eliminated from the intestine and liver circulates in the blood and can reach the brain, even if in smaller quantities compared to other organs, as recently demonstrated with mice subjected to a gavage of a mixture of ^{13}C fructose: glucose, followed by mass spectrometry to trace the fate of glucose and fructose carbons in vivo (Jang et al.,2018). In particular, fructose can be transported into the brain from the periphery through fructose-specific (GLUT5) or non-selective glucose and fructose transporters (GLUT2, GLUT8, GLUT9). GLUT5 is found on neurons (Kojo et al., 2016) and glia (Horikoshi et al., 2003; Kojo et al., 2016; Payne et al., 1997) within several brain regions, including the hippocampus (Kojo et al., 2016), cortex (Kojo et al., 2016; Oppelt et al., 2017), and hypothalamus (Kojo et al., 2016).
It has been shown that fructose consumption can increase the permeability of the blood brain barrier to facilitate fructose entry into the brain (Mamo et al., 2019; Takechi et al., 2017). Even as fructose is not the preferred energy substrate of the brain, and it alone is insufficient for neuronal survival (Rastedt et al., 2017), its presence in the brain suggests that brain cells can utilize fructose or that

fructose may influence cellular processes. Cells may use fructose to restore normal functions when glucose is depleted, thus fructose may serve as an alternative energy source during glucopenia (Thurston et al., 1972). Interestingly, the brain can also produce fructose from glucose via the polyol pathway under conditions of hyperglycemia (Hwang et al., 2017), aging (Schalkwijk et al.,2004) water restriction (Song et al., 2017), hyperosmolality, tissue hypoxia, heat stress, ischaemia, and hyperuricemia (R. J. Johnson et al.,2019). Furthermore, as the polyol pathway is linked to hyperglycemia and oxidative stress (Chung et al., 2003), fructose production in cells could have significant implications for cellular stress and dysfunction, especially in patients with diabetes (Asnaghi et al., 2003; Hwang et al., 2017). Of note, peripheral fructose production is hypothesized to have evolved as a survival mechanism against the lack of food, water, or oxygen (Johnson et al., 2020). These mechanisms that may have once supported survival may now contribute to the development of metabolic syndrome (Johnson et al., 2020) and other brain disorders such as Alzheimer's disease (Johnson et al., 2020) (Figure 1).

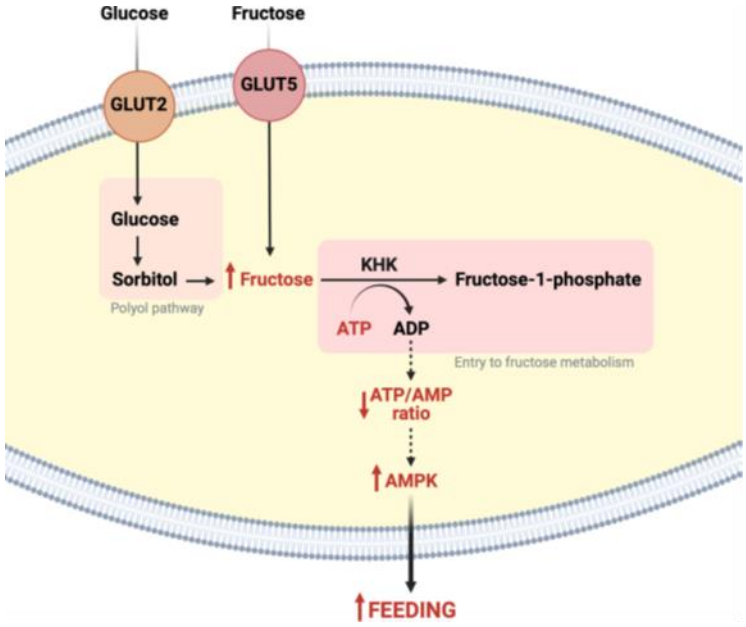

Figure 1. Intracellular mechanisms mediated by fructose. *Fructose can be transported into the cell via GLUT5, and fructose can also be synthesized inside the cell, especially during hyperglycemia when excess glucose builds up inside cells, through the polyol pathway. Within the cell, fructose is phosphorylated by ketohexokinase (KHK) into fructose-1-phosphate, and this phosphorylation depletes ATP levels. In effect, this reduces the ratio of ATP to AMP levels inside the cell and promotes the activation of AMP kinase (AMPK), which is associated with food intake in hunger-like states (Mikayla et al., 2021).*

Dietary fructose may exert direct or indirect actions on multiple brain systems to influence feeding behavior. In particular, fructose affects the release or response to hormones that regulate appetite and food intake at the level of the hypothalamus, which is the appetite center and the major energy status

sensor. Glucose is well-established to be a satiety signal that would suppress feeding (Page et al., 2013). Since fructose is a sugar, it might be thought that fructose would produce satiety to curb additional feeding, nevertheless, unlike glucose, fructose does not induce satiety. This is to be expected given that fructose does not stimulate satiety hormones such as leptin and insulin to the same degree as glucose (Luo et al., 2015; Page et al., 2013; Teff et al., 2004). In particular, fructose-induced health disturbances are different from those provoked by glucose or sucrose, likely because the initial steps of fructose metabolism differ from those of glucose not only in the liver but also in the central nervous system (CNS). In fact, fructose bypasses the rate-limiting regulatory step of glycolysis and is therefore metabolized far more rapidly than glucose. This leads in the hypothalamus to a rapid depletion of ATP (Cha et al., 2008), accompanied by an increase in AMP level, the activation of AMPK, the inactivation of acetyl-CoA carboxylase and a decrease of malonyl-CoA concentration, thus increasing food intake and risk of obesity (Cha et al., 2008; Lane et al.,2009).

Fructose and Neuroinflammation

Increasing evidence suggest that a fructose-rich diet is closely associated with chronic inflammation, characterized by an increased systemic inflammation (Porto et al., 2015), as well as inflammation signaling activation in local tissues and organs, including liver, adipose, kidney, heart and brain (Miller et al., 2008). The pro-inflammatory effect of fructose has been demonstrated in many brain regions, mainly in long dietary treatments with the sugar. Long-term fructose-drinking (sixteen weeks) caused neuroinflammation associated with impaired insulin signaling, oxidative stress, reduced activity of the cholinergic system and cognitive impairment in both the hippocampi and the cerebral cortices of rats (Yin et al., 2014). Fructose feeding induced increased expression of the proinflammatory mediator genes Interleukin (IL)-1beta, IL-6 and Tumor necrosis factor (TNF)-alpha, together with Toll-like receptor 4 (TLR4), myeloid differentiation factor 88 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), in the hypothalami of rats (Li et al., 2015). Fructose feeding was shown to be associated with increased levels of histone deacetylases 3 (HDAC3), thus suggesting that HDAC3 represents a crucial component in the network linking fructose to neuroinflammation in metabolic syndrome, through the activation of TLR4/NF-kB pathway (Li et al., 2015). Furthermore, excess consumption of HFCS exerted detrimental effects on hippocampal function and caused neuroinflammation in the CNS during adolescence (Hsu et al., 2015). Even a very short-term (seven days) fructose-rich diet was shown to induce morphologic, structural and functional modifications in a rat hippocampus, associated with an increase in reactive astrocytes and microglial activation (Ochoa et al.,2014). In contrast, Jimenez-Maldonado (Jiménez-Maldonado et al.,2018) advocated that a short period (just one week) of fructose can disturb neuronal

integrity and brain plasticity without inducing significant microglial activation. Although young and adults differ largely in their metabolic and physiological profiles, most of the mentioned studies investigated fructose-induced brain disturbances in adults. The research group where I did my PhD previously demonstrated that a short-term consumption of fructose (just two weeks) produces an early increase in specific markers of inflammation (TNF-alpha and glial fibrillary acidic protein) in the hippocampi of both young and adult rats (Cigliano et al., 2018). This diet also induced, in the frontal cortices of both age groups, the activation of autophagy, as well as imbalances in redox homeostasis (Figure 2) (Spagnuolo et al.,2020).

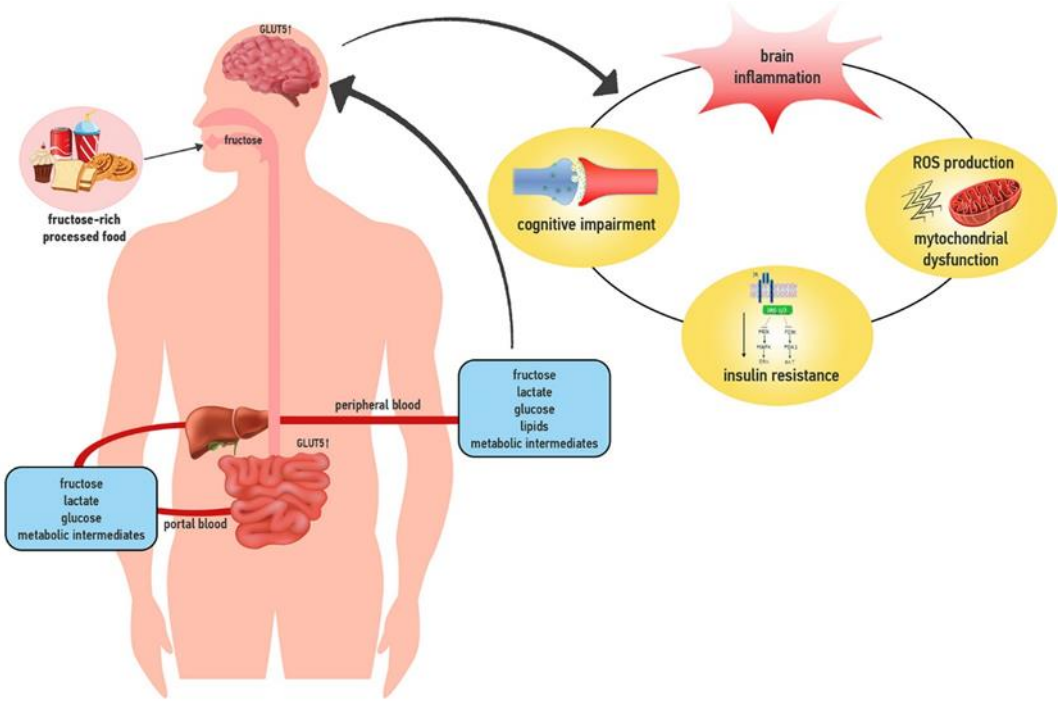

Figure 2. Fructose metabolism. *Fructose is cleared by the small intestine, which converts it into different metabolic intermediates. High fructose doses overwhelm the intestinal capacity for fructose metabolism, and a part of fructose spills over to the liver, which can metabolize it. Gut- and liver-derived metabolic intermediates or fructose itself can reach the brain, exerting effects on regulation of food intake, brain inflammation, mitochondrial function and oxidative stress, insulin signaling and cognitive function (Spagnuolo et al.,2020).*

Fructose and Oxidative stress

The brain is more susceptible to oxidative damage compared to other organs due to its high levels of oxygen consumption, its rich unsaturated fatty acids content and in iron catalysis (Dringen et al.,2000). Consequently, oxidative stress is a well-known factor associated to cognitive dysfunction in ageing and neurodegenerative disorders (Dringen et al.,2000). The proper brain mitochondrial function is crucial and mitochondrial ROS overproduction might be one of the leading causes of brain imbalances in redox homeostasis (Dringen et al.,2000; Schon et al.,2011). In fact, any impairment of

mitochondrial activity places neurons at a high risk of dysfunction and/or death (Wang et al.,2020). Although various studies evidenced the alteration of redox homeostasis induced by excessive fructose consumption at the systemic level (Crescenzo et al.,2013; Cioffi et al.,2017), only in the last decade the effects of this sugar on redox balance in the CNS have been explored. Interestingly, an increase in oxidative stress was found both after short-term and long-term fructose intake (Spagnuolo et al.,2020).The research group where I did my PhD demonstrated that a two-week fructose diet was associated with hippocampal oxidative stress, as increased levels of lipid and protein oxidation was detected in this brain area both in young and adult rats (Cigliano et al.,2018).The imbalance of redox homeostasis was also found in the frontal cortex of both fructose-fed young and adult rats, with lower amounts of Nrf2, lower activity of Glucose 6-phosphate dehydrogenase and Glutathione reductase and a lower Glutathione (GSH)/Oxidized Glutathione (GSSG) ratio (Spagnuolo et al.,2020). Since autophagy is usually activated as a protective mechanism in response to oxidative stress, we also showed for the first time that a short-term fructose-rich diet is associated with the activation of autophagy, as increased levels of beclin 1, microtubuleassociated protein 1A/1B-light chain 3 and phospho sequestosome 1 were detected in frontal cortices of rats of different ages (Spagnuolo et al.,2020). As for long-term fructose effects, rats receiving fructose supplementation (10% w/v in drinking water) for sixteen weeks showed significantly higher levels of ROS, lipid peroxides, carbonyls and lower levels of GSH compared to control rats in both the hippocampus and cerebral cortex, associated with lower activities of superoxide dismutase, catalase and glutathione peroxidase in both brain areas (Yin et al.,2014). Moreover, significant decrease in major markers of mitochondrial function has been detected in the brain after high fructose feeding in both short- and long-term experiments. In fact, a single week of fructose feeding induced a reduction of the coactivator gamma-1 alpha of the peroxisome proliferator-activated receptor (PGC1-) and of the subunit II of cytochrome c oxidase, indicative of mitochondrial dysfunction in the hippocampus (Jiménez-Maldonado et al., 2018). Furthermore, the data obtained from our research laboratory indicate that a high short-term fructose intake induces the production of advanced glycation end products (AGEs), in particular carboxymethyllysine and carboxyethyllisine, as well as a reduction in the activity of antioxidants enzymes (Spagnuolo et al.,2022; Mazzoli et al.,2021).

Fructose impact on cognitive function

Fructose consumption is currently perceived as an important cause of metabolic disorders with subsequent detriment of cognitive function. One of the first research studies evidencing the impact of fructose on learning and memory was performed on male Sprague Dawley rats fed a long-term (eight months), high-fat, high-glucose diet supplemented with 20% HFCS in the drinking water. This dietary

treatment was associated with a reduction in the hippocampal BDNF level and with the impairment of hippocampus-dependent learning and memory, synaptic plasticity and dendritic spine density (Stranahan et al.,2008). Recent studies have shown that the impact of fructose on cognitive function differs at different stages of life. Indeed, ad libitum consumption of an 11% HFCS-55 solution for thirty days in juvenile and adolescent, but not adult rats, was reported to impair hippocampal-dependent spatial learning and memory retention (Hsu et al.,2015). In agreement with these data, another research group demonstrated that only rats fed the high-fructose diet beginning at weaning showed increased anxiety-like and depressive-like behaviors in adulthood; rats that began the diet after reaching adulthood did not manifest the same behaviors (Harrell et al.,2015). In contrast, administration of a high-fructose diet (55% kcal from fructose) from adolescence until adulthood does not affect motor, anxiety-like, or depressive-like behavior in Sprague Dawley rats, nor did it modify the intrinsic excitability of basolateral amygdala principal neurons (O'Flaherty et al.,2019). These findings suggest that the effect of a fructose rich diet on affective behavior may be tightly dependent on strain and age. Short-term fructose feeding (10% w/v, fructose solution for five weeks) was shown to alter the sleep-wake cycle in young rats, eliciting wake-promoting effects and reducing the time spent in non-rapid eye movement sleep (Franco-Pérez et al.,2018). This effect was considered a consequence of the fructose-induced activation of orexinergic neurons in the hypothalamus and of dopaminergic neurons in midbrain regions (Franco-Pérez et al.,2018). Consumption of ad libitum drinking water containing 23% fructose over a short period (four weeks) was found to be associated with a reduction of neurogenesis and an increase of apoptosis in the dentate gyrus of the hippocampus (Van der Borght et al.,2011). The increase of apoptosis was ascribed to the observed increase in circulating TNF-alpha, while the reduced transport of ghrelin and leptin across the BBB was responsible for reduced hippocampal neurogenesis (Van der Borght et al.,2011). Experimental evidence in rodents shows that overconsumption of fructose (15% solutions) for six weeks, sufficient to disrupt peripheral metabolism, reduces hippocampal insulin receptor signaling, which is commensurable to poor learning and memory performance (Agrawal et al.,2016). In addition, two-month-old male C57BL/6 mice exposed to administration of normal food and water supplemented with 15% fructose for eight weeks lost their ability to sustain long-term hippocampal potentiation and long-term depression, showed a reduction in density and size of active zones at synapses, as well as decreased expression of the glutamate receptor subunits NMDAε1 and GluR2, thus indicating a deregulation of hippocampal synaptic transmission and plasticity. All of these changes are congruent with a reduction in learning and memory performance. (Cisternas et al.,2015). Finally, short-term (two weeks) high-fructose diet was shown to be associated with decreases in the presynaptic proteins synaptotagmin I, synapsin I, and synaptophysin I in juvenile rats, as well as

impairment of BDNF signaling, supporting the idea that the fructose-associated impairment of brain plasticity depends critically on the action of neurotrophin (Spagnuolo et al.,2020). In conclusion, although not all studies come to exactly the same conclusion, enough evidence has accumulated over the past ten years to indicate that fructose intake, in certain doses, has a significant impact on cognitive function.

References

1. R. Agrawal, E. Noble, L. Vergnes, Z. Ying, K. Reue, F. Gomez-Pinilla, Dietary fructose aggravates the pathobiology of traumatic brain injury by influencing energy homeostasis and plasticity, Journal of Cerebral Blood Flow and Metabolism 36 (2016) 941–953.

2. Alwahsh, S.M.; Gebhardt, R. Dietary fructose as a risk factor for non-alcoholic fatty liver disease (NAFLD). Arch. Toxicol. 2017,91, 1545–1563.

3. Asnaghi, V., Gerhardinger, C., Hoehn, T., Adeboje, A., & Lorenzi, M. (2003). A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. Diabetes, 52(2), 506-511.

4. Azais-Braesco V, Sluik D, Maillot M, Kok F, Moreno LA. A review of total & added sugar intakes and dietary sources in Europe. Nutr J. 2017;16:6.

5. Bray, G.A. Soft drink consumption and obesity: It is all about fructose. Curr. Opin. Lipidol. 2010, 21, 51–57.

6. Bray, G.A.; Nielsen, S.J.; Popkin, B.M. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. Am. J. Clin. Nutr. 2004, 79, 537–543.

7. Cha, S.H.;Wolfgang, M.; Tokutake, Y.; Chohnan, S.; Lane, M.D. Differential effects of central fructose and glucose on hypothalamic malonyl-coa and food intake. Proc. Natl. Acad. Sci. USA 2008, 105, 16871–16875.

8. Chung SS, Ho EC, Lam KS, Chung SK (2003) Contribution of polyol pathway to diabetes-induced oxidative stress. J Am Soc Nephrol 14:S233–6

9. Cigliano, L.; Spagnuolo, M.S.; Crescenzo, R.; Cancelliere, R.; Iannotta, L.; Mazzoli, A.; Liverini, G.; Iossa, S. Short-Term Fructose Feeding Induces Inflammation and Oxidative Stress in the Hippocampus of Young and Adult Rats. Mol. Neurobiol. 2018,55, 2869–2883.

10. Cioffi, F.; Senese, R.; Lasala, P.; Ziello, A.; Mazzoli, A.; Crescenzo, R.; Liverini, G.; Lanni, A.; Goglia, F.; Iossa, S. Fructose-Rich Diet Affects Mitochondrial DNA Damage and Repair in Rats. Nutrients 2017, 9, 323.

11. P. Cisternas, P. Salazar, F.G. Serrano, C. Montecinos-Oliva, S.B. Arredondo, L. Varela-Nallar, S. Barja, C.P. Vio, F. Gomez-Pinilla, N.C. Inestrosa, Fructose consumption reduces hippocampal synaptic plasticity underlying cognitive performance, Biochim. Biophys. Acta 1852 (2015) 2379–2390

12. Crescenzo, R.; Bianco, F.; Coppola, P.; Mazzoli, A.; Cigliano, L.; Liverini, G.; Iossa, S. Increased skeletal muscle mitochondrial efficiency in rats with fructose-induced alteration in glucose tolerance. Br. J. Nutr. 2013, 110, 1996–2003.

13. Dringen R, Gutterer JM, Hirrlinger J. Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. European journal of biochemistry / FEBS. 2000;267:4912 -6.

14. Faeh, D., Minehira, K., Schwarz, J.M., Periasamy, R., Park, S., Tappy, L., 2005. Effect of fructose overfeeding and fish oil administration on hepatic de novo lipogenesis and insulin sensitivity in healthy men. Diabetes 54, 1907–1913.

15. Fields, S., 2004. The fat of the land: do agricultural subsidies foster poor health? Environ. Health Perspect. 112, A820–A823.

16. Franco-Pérez, J.; Manjarrez-Marmolejo, J.; Ballesteros-Zebadúa, P.; Neri-Santos, A.; Montes, S.; Suarez-Rivera, N.; Hernández-Cerón, M.; Pérez-Koldenkova, V. Chronic Consumption of Fructose Induces Behavioral Alterations by Increasing Orexin and Dopamine Levels in the Rat Brain. Nutrients 2018, 10, 1722, doi: 10.3390/nu10111722.

17. Goran, M.I., Ulijaszek, S.J., Ventura, E.E., 2013b. High fructose corn syrup and diabetes prevalence: a global perspective. Glob. Public Health 8, 55–64.

18. Harrell, C.S.; Burgado, J.; Kelly, S.D.; Johnson, Z.P.; Neigh, G.N. High-fructose Diet During Periadolescent Development Increases Depressive-Like Behavior and Remodels the Hypothalamic Transcriptome in Male Rats. Psychoneuroendocrinology 2015, 62, 252–264, doi:10.1016/j.psyneuen.2015.08.025.

19. Horikoshi, Y., Sasaki, A., Taguchi, N., Maeda, M., Tsukagoshi, H., Sato, K., & Yamaguchi, H. (2003). Human GLUT5 immunolabeling is useful for evaluating microglial status in neuropathological study using paraffin sections. *Acta neuropathologica*, *105*(2).

20. Hsu, T.M.; Konanur, V.R.; Taing, L.; Usui, R.; Kayser, B.D.; Goran, M.I.; Kanoski, S.E. Effects of sucrose and high fructose corn syrup consumption on spatial memory function and hippocampal neuroinflammation in adolescent rats. Hippocampus 2015, 25, 227–239.

21. Hwang, J. J., Jiang, L., Hamza, M., Dai, F., Belfort-DeAguiar, R., Cline, G., ... & Sherwin, R. S. (2017). The human brain produces fructose from glucose. *JCI insight*, *2*(4).

22. Jang, C., Hui, S., Lu, W., Cowan, A.J., Morscher, R.J., Lee, G., Liu, W., Tesz, G.J., Birnbaum, M.J., Rabinowitz, J.D., 2018. The small intestine converts dietary fructose into glucose and organic acids. Cell Metab. 27, 351–361.

23. Jiménez-Maldonado, A.; Ying, Z.; Byun, H.R.; Gomez-Pinilla, F. Short-term fructose ingestion affects the brain independently from establishment of metabolic syndrome. Biochim. Biophys. Acta Mol. Basis Dis. 2018, 1864, 24–33.

24. R. J. Johnson, P. Stenvinkel, P. Andrews, L. G. Sánchez-Lozada, T. Nakagawa, E. Gaucher, A. Andres-Hernando, B. Rodriguez-Iturbe, C. R. Jimenez, G. Garcia, D.-H. Kang, D. R. Tolan, M. A. Lanaspa. Fructose metabolism as a common evolutionary pathway of survival associated with climate change, food shortage and droughts. J Intern Med 2020 Mar;287(3):252-262. doi: 10.1111/joim.12993.

25. Johnson, R. J., Gomez-Pinilla, F., Nagel, M., Nakagawa, T., Rodriguez-Iturbe, B., Sanchez-Lozada, L. G., ... & Lanaspa, M. A. (2020). Cerebral fructose metabolism as a potential mechanism driving Alzheimer's disease. Frontiers in aging neuroscience, 12, 560865.

26. Kojo A, Yamada K, Yamamoto T. Glucose transporter 5 (GLUT5)-like immunoreactivity is localized in subsets of neurons and glia in the rat brain. J Chem Neuroanat. 2016 Jul;74:55-70. doi: 10.1016/j.jchemneu.2016.03.004. Epub 2016 Mar 29. PMID: 27036089.

27. Lane, M.D.; Cha, S.H. Effect of glucose and fructose on food intake via malonyl-CoA signaling in the brain. Biochem. Biophys. Res. Commun. 2009, 382, 1–5.

28. Li, J.M.; Ge, C.X.; Xu, M.X.;Wang,W.; Yu, R.; Fan, C.Y.; Kong, L.D. Betaine recovers hypothalamic neural injury by inhibiting astrogliosis and inflammation in fructose-fed rats. Mol. Nutr. Food Res. 2015, 59, 189–202.

29. Livesey, G.; Taylor, R. Fructose consumption and consequences for glycation, plasma triacylglycerol, and body weight: Metaanalyses and meta-regression models of intervention studies. Am. J. Clin. Nutr. 2008, 88, 1419–1437.

30. Luo, S., Monterosso, J. R., Sarpelleh, K., & Page, K. A. (2015). Differential effects of fructose versus glucose on brain and appetitive responses to food cues and decisions for food rewards. Proceedings of the National Academy of Sciences, 112(20), 6509-6514.

31. Mamo JC, Lam V, Brook E, Mooranian A, Al-Salami H, Fimognari N, Nesbit M, Takechi R. Probucol prevents blood-brain barrier dysfunction and cognitive decline in mice maintained on pro-diabetic diet. Diab Vasc Dis Res. 2019 Jan;16(1):87-97. doi: 10.1177/1479164118795274. Epub 2018 Aug 29. PMID: 30156119.

32. Mazzoli A, Spagnuolo MS, Nazzaro M, Gatto C, Iossa S, Cigliano L (2021) Fructose removal from the diet reverses inflammation, mitochondrial dysfunction, and oxidative stress in hippocampus. Antioxidants (Basel) 10(3):487. https:// doi. org/ 10. 3390/ antio x1003 048729

33. Mikayla A. Payant, Melissa J. Chee. Neural mechanisms underlying the role of fructose in overfeeding. Neuroscience and Biobehavioral Reviews 128 (2021) 346–357.

34. Miller, A.; Adeli, K. Dietary fructose and the metabolic syndrome. Curr. Opin. Gastroenterol. 2008, 24, 204–209.

35. Ochoa, M.; Malbert, C.H.; Lallès, J.P.; Bobillier, E.; Val-Laillet, D. Effects of chronic intake of starch-, glucose- and fructosecontaining diets on eating behaviour in adult minipigs. Appl. Anim. Behav. Sci. 2014, 157, 61–71.

36. O'Flaherty, B.; Neigh, G.N.; Rainnie, D. High-fructose diet initiated during adolescence does not affect basolateral amygdala excitability or affective-like behavior in Sprague Dawley rats. *Behav. Brain. Res.* 2019, *365*, 17–25, doi:10.1016/j.bbr.2019.02.042

37. Oppelt SA, Zhang W, Tolan DR. Specific regions of the brain are capable of fructose metabolism. Brain Res. 2017 Feb 15;1657:312-322. doi: 10.1016/j.brainres.2016.12.022. Epub 2016 Dec 27. PMID: 28034722; PMCID: PMC5420427.

38. Page, K. A., Chan, O., Arora, J., Belfort-DeAguiar, R., Dzuira, J., Roehmholdt, B., ... & Sherwin, R. S. (2013). Effects of fructose vs glucose on regional cerebral blood flow in brain regions involved with appetite and reward pathways. *Jama*, *309*(1), 63-70.

39. Payne J, Maher F, Simpson I, Mattice L, Davies P. Glucose transporter Glut 5 expression in microglial cells. Glia. 1997 Nov;21(3):327-31. doi: 10.1002/(sici)1098-1136(199711)21:3<327::aid-glia7>3.0.co;2-1. PMID: 9383041.

40. Porto, M.L.; Lirio, L.M.; Dias, A.T.; Batista, A.T.; Campagnaro, B.P.; Mill, J.G.; Meyrelles, S.S.; Baldo, M.P. Increased oxidative stress and apoptosis in peripheral blood mononuclear cells of fructose-fed rats. Toxicol. In Vitro 2015, 29, 1977–1981.

41. Rastedt DE, Vaughan RA, Foster JD, 2017 Palmitoylation mechanisms in dopamine transporter regulation. J Chem Neuroanat 83–84, 3–9.

42. Samuel, V.T., 2011. Fructose induced lipogenesis: from sugar to fat to insulin resistance. Trends Endocrinol. Metab. 22, 60–65.

43. Schon EA, Przedborski S. Mitochondria: the next (neurode)generation. Neuron. 2011;70:1033 -53.

44. Schulze, M.B.; Manson, J.E.; Ludwig, D.S.; Colditz, G.A.; Stampfer, M.J.; Willett, W.C.; Hu, F.B. Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. JAMA 2004, 292, 927–934.

45. Schalkwijk CG, Stehouwer CD, van Hinsbergh VW. Fructose-mediated non-enzymatic glycation: sweet coupling or bad modification. Diabetes Metab Res Rev. 2004 Sep-Oct;20(5):369-82. doi: 10.1002/dmrr.488. PMID: 15343583.

46. Schwarz, J.M., Noworolski, S.M., Wen, M.J., Dyachenko, A., Prior, J.L., Weinberg, M.E., Herraiz, L.A., Tai, V.W., Bergeron, N., Bersot, T.P., Rao, M.N., Schambelan, M., Mulligan, K., 2015. Effect of a high-fructose weight-maintaining diet on lipogenesis and liver fat. J. Clin. Endocrinol. Metab. 100, 2434–2442.

47. Shapiro, A.; Mu,W.; Roncal, C.; Cheng, K.Y.; Johnson, R.J.; Scarpace, P.J. Fructose-induced leptin resistance exacerbates weight gain in response to subsequent high-fat feeding. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2008, 295, R1370–R1375.

48. Smith, S.M., 1988. High-fructose corn syrup replaces sugar in processed foods. Environ.Nutr. 11, 7–8.

49. Song Z, Roncal-Jimenez CA, Lanaspa-Garcia MA, Oppelt SA, Kuwabara M, Jensen T, Milagres T, Andres-Hernando A, Ishimoto T, Garcia GE, Johnson G, MacLean PS, Sanchez-Lozada LG, Tolan DR, Johnson RJ. Role of fructose and fructokinase in acute dehydration-induced vasopressin gene expression and secretion in mice. J Neurophysiol. 2017 Feb 1;117(2):646-654. doi: 10.1152/jn.00781.2016. Epub 2016 Nov 16. PMID: 27852737; PMCID: PMC5288484.

50. Spagnuolo, M.S.; Bergamo, P.; Crescenzo, R.; Iannotta, L.; Treppiccione, L.; Iossa, S.; Cigliano, L. Brain Nrf2 Pathway, Autophagy, and Synaptic Function Proteins Are Modulated by a Short-Term Fructose Feeding in Young and Adult Rats. Nutr. Neurosci. 2020, 23, 309–320.

51. Spagnuolo MS, Iossa S, Cigliano L. Sweet but Bitter: Focus on Fructose Impact on Brain Function in Rodent Models. Nutrients. 2020 Dec 22;13(1):1. doi: 10.3390/nu13010001. PMID: 33374894; PMCID: PMC7821920.

52. Spagnuolo, M.S., Mazzoli, A., Nazzaro, M., Troise, A.D., Gatto, C., Tonini, C., Colardo, M., Segatto, M., Scaloni, A., Pallottini, V., Iossa, S., Cigliano, L. Long-Lasting Impact of Sugar Intake

on Neurotrophins and Neurotransmitters from Adolescence to Young Adulthood in Rat Frontal Cortex. Mol Neurobiol. 2022. doi: 10.1007/s12035-022-03115-8.

53. Stanhope, K.L. Role of fructose-containing sugars in the epidemics of obesity and metabolic syndrome. Annu. Rev. Med. 2012, 63, 329–343.

54. Stanhope, K.L., 2015. Sugar consumption, metabolic disease and obesity: the state of the controversy. Crit. Rev. Clin. Lab. Sci. 53, 1–16.

55. Stanhope, K.L., Schwarz, J.M., Keim, N.L., Griffen, S.C., Bremer, A.A., Graham, J.L., Hatcher, B., Cox, C.L., Dyachenko, A., Zhang, W., McGahan, J.P., Seibert, A., Krauss, R.M., Chiu, S., Schaefer, E.J., Ai, M., Otokozawa, S., Nakajima, K., Nakano, T., Beysen, C., Hellerstein, M.K., Berglund, L., Havel, P.J., 2009. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. J. Clin. Invest. 119, 1322–1334.

56. Steenson, S., Shojaee-Moradie, F., B Whyte, M., G Jackson, K., Lovegrove, J.A., A Fielding, B., Umpleby, A.M., 2020. The effect of fructose feeding on intestinal triacylglycerol production and de novo fatty acid synthesis in humans. Nutrients 12, 1781.

57. Stranahan, A.M.; Norman, E.D.; Lee, K.; Cutler, R.G.; Telljohann, R.S.; Egan, J.M.; Mattson, M.P. Diet-induced Insulin Resistance Impairs Hippocampal Synaptic Plasticity and Cognition in Middle-Aged Rats. *Hippocampus* 2008, *18,* 1085–1088, doi:10.1002/hipo.20470

58. Takechi, R., Lam, V., Brook, E., Giles, C., Fimognari, N., Mooranian, A., ... & Mamo, J. C. (2017). Blood-brain barrier dysfunction precedes cognitive decline and neurodegeneration in diabetic insulin resistant mouse model: an implication for causal link. Frontiers in aging neuroscience, 9, 399.

59. Taskinen, M.R., Packard, C.J., Boren, J., 2019. Dietary fructose and the metabolic syndrome. Nutrients 11, 1987.

60. Teff, K. L., Grudziak, J., Townsend, R. R., Dunn, T. N., Grant, R. W., Adams, S. H., ... & Havel, P. J. (2009). Endocrine and metabolic effects of consuming fructose-and glucose-sweetened beverages with meals in obese men and women: influence of insulin resistance on plasma triglyceride responses. *The Journal of Clinical Endocrinology & Metabolism*, *94*(5), 1562-1569.

61. Theytaz, F., de Giorgi, S., Hodson, L., Stefanoni, N., Rey, V., Schneiter, P., Giusti, V., Tappy, L., 2014. Metabolic fate of fructose ingested with and without glucose in a mixed meal. Nutrients 6, 2632–2649.

62. Thurston, J. H., Levy, C. A., Warren, S. K., & Jones, E. M. (1972). Permeability of the blood-brain barrier to fructose and the anaerobic use of fructose in the brains of young mice 1. Journal of neurochemistry, *19*(7), 1685-1696.

63. K. van der Borght, R. Kohnke, N. Goransson, T. Deierborg, P. Brundin, C. Erlanson-Albertsson, A. Lindqvist, Reduced neurogenesis in the rat hippocampus following high fructose consumption, Regul. Pept. 167 (2011) 26–30.

64. Vos, M.B., Kimmons, J.E., Gillespie, C., Welsh, J., Blanck, H.M., 2008. Dietary fructose consumption among US children and adults: the third national health and nutrition examination survey. Medscape J. Med. 10, 160.

65. Wang, W.; Zhao, F.; Ma, X.; Perry, G.; Zhu, X. Mitochondria dysfunction in the pathogenesis of Alzheimer's disease: Recent advances. Mol. Neurodeg. 2020, 15, 30.

66. Yin, Q.; Ma, Y.; Hong, Y.; Hou, X.; Chen, J.; Shen, C.; Sun, M.; Shang, Y.; Dong, S.; Zeng, Z.; et al. Lycopene attenuates insulin signaling deficits, oxidative stress, neuroinflammation, and cognitive impairment in fructose-drinking insulin resistant rats. Neuropharmacology 2014, 86, 389–396.

67. Zhang DM, Jiao RQ, Kong LD. High Dietary Fructose: Direct or Indirect Dangerous Factors Disturbing Tissue and Organ Functions. Nutrients. 2017 Mar 29;9(4):335.

Chapter 2

Fructose Removal from the Diet Reverses Inflammation, Mitochondrial Dysfunction, and Oxidative Stress in Hippocampus

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Fructose Removal from the Diet Reverses Inflammation, Mitochondrial Dysfunction, and Oxidative Stress in Hippocampus

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Abstract: Young age is often characterized by high consumption of processed foods and fruit juices rich in fructose, which, besides inducing a tendency to become overweight, can promote alterations in brain function. The aim of this study was therefore to (a) clarify brain effects resulting from fructose consumption in juvenile age, a critical phase for brain development, and (b) verify whether these alterations can be rescued after removing fructose from the diet. Young rats were fed a fructose-rich or control diet for 3 weeks. Fructose-fed rats were then fed a control diet for a further 3 weeks. We evaluated mitochondrial bioenergetics by high-resolution respirometry in the hippocampus, a brain area that is critically involved in learning and memory. Glucose transporter-5, fructose and uric acid levels, oxidative status, and inflammatory and synaptic markers were investigated by Western blotting and spectrophotometric or enzyme-linked immunosorbent assays. A short-term fructose-rich diet induced mitochondrial dysfunction and oxidative stress, associated with an increased concentration of inflammatory markers and decreased Neurofilament-M and post-synaptic density protein 95. These alterations, except for increases in haptoglobin and nitrotyrosine, were recovered by returning to a control diet. Overall, our results point to the dangerous effects of excessive consumption of fructose in young age but also highlight the effect of partial recovery by switching back to a control diet.

Keywords: hippocampus; mitochondria; fructose diet; young rat; inflammation; oxidative stress; haptoglobin; neurofilament-M; PSD-95

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1. Introduction

In previous decades, a significant increase in the fructose content of the human diet has occurred, far above what is introduced daily with fruits and vegetables, because of the increased consumption of industrial foods and the extensive commercial use of high-fructose corn syrup (HFCS) as a sweetener for beverages, coffee, snacks, and bakery foods [1,2]. High fructose consumption has long been known to expose the consumer to metabolic health risks, such as obesity, lipid alterations, insulin resistance, and inflammation [3–6].

Recently, different lines of evidence in animal models highlighted the marked effects of this sugar on brain structure and function [7,8]. In fact, long-term feeding with fructose can cause brain alterations such as insulin signaling [9], neurogenesis [10], and redox homeostasis, as well as neuroinflammation [11,12]. Further experimental studies have highlighted possible mechanisms at the basis of cognitive deficits induced by the consumption of this sugar [13–15]. We also reported that a short-term fructose diet induced neuroinflammation, oxidative stress, and impairment of insulin signaling in the hippocampi of young and adult

rats [16]. It has been shown that just a week of fructose ingestion negatively impacts brain plasticity [17]. Interestingly, a very recent study showed that the spatial memory deficit, neuroinflammation, and brain protein changes induced by 12 weeks of fructose drinking in adult rats persisted for one month after its elimination from the diet [18].

Young people are among the highest consumers of sweet foods, especially fructose-sweetened beverages [19]. Metabolic risk factors related to poor nutrition can arise at a very early age; hence, investigations are required to clarify the brain consequences resulting from a diet rich in fructose at a critical stage of development. Indeed, despite the importance of this issue, few studies have been performed in rodents, providing evidence that, in childhood and adolescence, critical periods of neurocognitive development, the impact of high dietary fructose consumption on hippocampal function is particularly damaging [16,20]. Of note, a very recent study of neuroimaging on 103 healthy children aged 7 to 11 years supported animal data and translated these findings to humans, providing evidence that increases in dietary fructose are associated with alterations in hippocampal structure and connectivity [21].

Given the limited research on the persistence of the brain alterations caused by high fructose consumption in individuals of juvenile age, in this study we focused our analysis on the hippocampi of young (30 days old) rats fed a fructose-rich (F) or control diet (C) for 3 weeks. After this period, fructose-fed rats were fed a control diet for a further 3 weeks (FR) and compared with rats that received the control diet for the entire period (CR) in order to clarify whether alterations induced by a short-term fructose diet can be rescued after removing fructose from the diet. Attention was focused on the hippocampus, a brain area responsible for learning, memory, and food intake regulation that is particularly susceptible to dietary and metabolic insults [20,22,23]. This investigation aimed to highlight the early effects (3 weeks of treatment) of the fructose diet on hippocampal expression of glucose transporter-5 (Glut-5), fructose and uric acid levels, inflammation and redox homeostasis in terms of mitochondrial function, and oxidative damage to lipids and proteins as well as antioxidant enzyme activities. In addition, as alterations to redox homeostasis are closely connected with changes in neuronal function, the effects of dietary fructose treatment on neurofilament-M (NF-M), synaptophysin, synaptotagmin, and post-synaptic density protein 95 (PSD 95) were also investigated.

2. Materials and Methods

2.1. Materials

Goat anti-Rabbit-Horseradish peroxidase-conjugated IgGs (GAR-HRP) and Goat anti-Mouse-Horseradish peroxidase-conjugated IgGs (GAM-HRP) were purchased from Immunoreagent (Raleigh, NC, USA). Fuji Super RX 100 films (Laboratorio Elettronico Di Precisione, Naples, Italy), polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Milan, Italy), and dye reagent for protein titration (Bio-Rad, Hercules, CA, USA) were used for Western blotting. Salts, buffers, and Bovine serum albumin fraction V (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Experimental Design

Male Wistar rats (Charles River, Italy), 30 days of age, were purchased from Charles River (Calco, Como, Italy) and housed as previously described [16]. Treatment, management, and euthanasia of animals met the guidelines set out by the Italian Health Ministry, and all experimental procedures were approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II (448/2019-PR).

The rats were divided into two groups, one fed a fructose rich diet (F group) and the other fed a control diet (C group) for 3 weeks. The control and fructose-rich diet compositions are reported in Table 1. At the end of the 3-week period, half of the rats from each group were euthanized and the other half were maintained on a control diet (FR and CR groups) for a further 3 weeks. Body weight and food and water intake were monitored daily. At the end of the experimental period, the animals were euthanized and

decapitated. The hippocampi were harvested and dissected [24]. Mitochondrial oxygen consumption was immediately assessed in little sections of tissue. Pieces of each sample were fixed for immunofluorescence analysis, and the remaining samples were snap frozen in liquid nitrogen and stored at −80 °C.

Table 1. Ingredients and nutritional composition of experimental diets.

Ingredients, g/100 g	Control Diet	Fructose Diet
Standard Chow [a]	50.5	50.5
Sunflower Oil	1.5	1.5
Casein	9.2	9.2
Alphacel	9.8	9.8
Cornstarch	20.4	-
Fructose	-	20.4
Water	6.4	6.4
AIN-76 mineral mix	1.6	1.6
AIN-76 vitamin mix	0.4	0.4
Choline	0.1	0.1
Methionine	0.1	0.1
Energy content and composition		
Gross Energy Density (kJ/g)	17.2	17.2
ME content (kJ/g) [b]	11.1	11.1
Proteins (% ME)	29.0	29.0
Lipids (% ME)	10.6	10.6
Carbohydrates (% ME)	60.4	60.4
Of which		
Fructose	-	30.0
Starch	52.8	22.8
Sugars	7.6	7.6

[a] 4RF21, Mucedola, Italy; [b] Estimated by computation using values (kJ/g) for energy content as follows: proteins 16.736, lipids 37.656, and carbohydrates 16.736. ME = metabolizable energy.

2.3. Metabolic Parameters

Colorimetric enzymatic methods were used to assess the contents of fructose and uric acid in the hippocampus using commercial kits (Sigma Aldrich, St. Louis, MO, USA for fructose, and GS Diagnostics SRL, Guidonia Montecelio, Rome, Italy for uric acid).

2.4. Protein Extraction

Aliquots of frozen hippocampus (about 80 mg) were homogenized in six volumes (*w*/*v*) of cold RIPA buffer, as previously described [25,26]. The protein concentration was measured with colorimetric Bio-Rad Bradford Protein Assays using a commercial kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Selected markers of inflammation (TNF-alpha; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-kB; ionized calcium binding adapter 1, Iba 1; glial fibrillary acidic protein, GFAP; Haptoglobin, Hpt), synaptic function (synaptophysin, synaptotagmin I, post synaptic density protein 95, PSD 95), and protein oxidative damage (Nitro-Tyrosine) were then evaluated by Western blotting or ELISA, as described below.

2.5. Analysis of Tumor Necrosis Factor Alpha (TNF-Alpha)

The TNF-alpha Duo-Set kit (R&D, DBA Italia) was used to measure the TNF-alpha concentration by sandwich ELISA, as previously described [27]. Data are expressed in pg of TNF-alpha per mg of proteins (assessed by Bradford Protein assay).

2.6. Western Blotting

Aliquots (30 µg of protein) of hippocampus were analyzed by electrophoresis in denaturing and reducing conditions [28] on 12.5% (to quantify Glut-5, Iba 1, GFAP, synaptophysin, adiponectin) or 10% (NF-kB, PSD 95, synaptotagmin I) polyacrylamide gel. Proteins

were transferred onto PVDF membrane [29], which was then washed by T-TBS (130 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.4). The membrane was incubated (60 min, 37 °C) in T-TBS containing 5% non-fat milk or 3% BSA (blocking step), and then the following detections were performed: Glut-5 by rabbit anti Glut-5 IgG (Invitrogen, Carlsbad, CA, USA, 0.5 µg/mL in T-TBS containing 3% BSA; overnight, 4 °C), followed by GAR-HRP IgG (1:45,000 dilution; 1 h, 37 °C); adiponectin by rabbit anti-human adiponectin IgG (Immunological Sciences, Rome, Italy; 1:500 dilution in in T-TBS containing 3% BSA; overnight, 4 °C), followed by GAR-HRP IgG (1:15,000 dilution; 1 h, 37 °C); peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) by rabbit anti-human PGC-1 IgG (Millipore, Burlington, MA, USA; 1:2000 in in T-TBS containing 3% BSA; overnight, 4 °C) with), followed by GAR-HRP IgG (1:20,000 dilution; 1 h, 37 °C); p-NF-kB and NF-kB by monoclonal mouse antibodies (Santa Cruz biotechnology, Dallas, TX, USA; 1:200 and 1:500, respectively, in 3% BSA; overnight, 4 °C), followed by GAM-HRP IgG (1:50,000 and 1:15,000, respectively, in 3% BSA; 1 h, 37 °C); Iba 1 by anti-Iba1 (1022-5) IgG (Santa Cruz biotechnology; 1: 500 in 3% BSA; overnight, 4 °C) and then GAM-HRP (1:10,000 in 1% non-fat milk; 1 h, 37 °C); GFAP by rabbit anti-human GFAP IgG (Cell Signaling, Beverly, MA, USA; 1:1000 in 1% non-fat milk; overnight, 4 °C), followed by GAR-HRP IgG (1:150,000 in 5% non-fat milk; 1 h, 37 °C); synaptophysin by anti-synaptophysin IgG (Merk Millipore, Milan, Italy; 1:150,000 in 3% BSA; overnight, 4 °C), followed by GAR-HRP IgG (1:20,000 dilution in 1% non-fat milk; 1 h, 37 °C); synaptotagmin I by anti-synaptotagmin I IgG (Cell Signaling; 1:1000 in 3% non-fat milk; overnight, 4 °C) followed by GAR-HRP IgG (1:100,000 dilution in 3% non-fat milk; 1 h, 37 °C); and PSD 95 by anti-PSD 95 IgG (Cell Signaling; 1:1000 in 3% non-fat milk; overnight, 4 °C), followed by GAR-HRP IgG (1:70,000 dilution in 3% non-fat milk; 1 h, 37 °C).

To load the control, β-actin was revealed after the detection of each marker. For this aim, the membranes were stripped [27] and then treated with mouse anti-β-actin IgG (1000 in 0.25% non-fat milk; overnight, 4 °C), followed by GAM-HRP IgG (1:30,000 in 0.25% non-fat milk; 1 h, 37 °C). The Excellent Chemiluminescent detection kit (Cyanagen s.r.l., Bologna, Italy) was used for detection. Chemidoc or digital images of X-ray films exposed to immunostained membranes were used for the densitometric analysis, and quantification was carried out with Un-Scan-It gel software (Silk Scientific, Orem, UT, USA).

2.7. Haptoglobin (Hpt) and Hemoglobin (Hb) Evaluation

Hpt concentration in hippocampus samples was measured by ELISA using rabbit anti-human haptoglobin (Sigma-Aldrich, St. Louis, MO, USA) in accordance with Mazzoli et al., 2020 [30].

The Hb concentration was measured by ELISA in hippocampus samples (diluted 1:200, 1:800, 1:1500) using rabbit anti-human Hb IgG (1: 500 dilution in T-TBS containing 0.25% BSA; Sigma-Aldrich, St. Louis, MO, USA), followed by 60 µL of GAR-HRP IgG (1:5000 dilution, 1 h, 37 °C). Coating, washing, and blocking were carried out as previously reported [31].

2.8. Mitochondrial Analyses in Rat Hippocampi

Hippocampus samples were homogenized (1:1000, *w*/*v*) in Mir05 medium (110 mM sucrose, 60 mM K-lactobionate, 20 mM Hepes, 20 mM taurine, 10 mM KH2PO4, 6 mM $MgCl_2$, 0.5 mM EGTA, 0.1% fatty acid free BSA, pH 7.0).

Aliquots of homogenates (2 mg) were used to measure the oxygen flux (pmol O2 s^{-1} mL^{-1}) with an Oxygraph-2k (O2k, OROBOROS INSTRUMENTS, Innsbruck, Austria) at 37 °C.

A substrate, uncoupler, inhibitor titration (SUIT) protocol was applied to assess qualitative and quantitative mitochondrial changes [32]. Leak respiration (CI_L) through complex I (CI) was evaluated using malate (0.5 mM), pyruvate (5 mM), and glutamate (10 mM). Phosphorylating respiration supported by complex I (CI_P) was assessed by the addition of 2.5 mM ADP. Respiration supported by complexes I and II ($C_{I\&IIP}$) was measured by adding 10 mM of succinate. Oligomycin was added at a concentration of 2.5 mM to assess leak respi-

ration ($C_{I\&IIL}$). The maximum capacity of the electron transport chain ($C_{I\&IIE}$) was obtained by the addition of the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 0.5 mM). Rotenone (0.5 µM) was added to determine the maximal capacity supported by CII alone d (C_{IIE}). The residual oxygen consumption was established by the addition of the inhibitor antimycin A (2.5 mM), and the resulting value was subtracted from the flux in each run to correct for nonmitochondrial respiration.

Calculation of Intrinsic Mitochondrial Function By using high resolution respirometry (HRR), we were able to assess different respiratory states within the same sample [33–35]. From these respiratory states, flux control ratios (FCR) could be calculated for leak respiration (FCR_L) with electron provision from complex I or complexes I and II and phosphorylating respiration (FCR_P) with electron provision from complex I or complexes I and II by using the formulas detailed below:

$FCR_{LI} = CI_L/C_{I\&IIE;}$
$FCR_{LI\&II} = C_{I\&IIL}/C_{I\&IIE;}$
$FCR_{PI} = CI_P/C_{I\&IIE};$
$FCR_{PI\&II} = C_{I\&IIP}/C_{I\&IIE.}$
In addition, flux control factors (FCFs) were calculated as follows:
Coupling efficiency of oxidative phosphorylation $= 1 - C_{I\&IIL}/C_{I\&IIP};$
Excess capacity of the electron transport chain $= 1 - C_{I\&IIP}/C_{I\&IIE};$
FCF Complex I $= 1 - (C_{IIE}/C_{I\ \&IIE});$
FCF Complex II $= 1 - (CI_P/C_{I\ +\ IIP})$.

Procedures to test mitochondrial integrity were routinely carried out at the beginning of each measurement by measuring the effect of 10 mM of exogenous cytochrome c on complex I-supported mitochondrial respiration.

2.9. Oxidative Stress Markers and Antioxidant Enzymes

Nitro-tyrosine (N-Tyr) titration was carried out by ELISA in hippocampal homogenates (diluted 1:1500, 1:3000, 1:6000) using rabbit anti-N-Tyr IgG (Covalab, distributed by VinciBiochem, Vinci, Italy; 1:600 dilution in T-TBS containing 0.25% BSA), followed by 60 µL of GAR-HRP (1:3500 dilution; 1 h, 37 °C), essentially as previously described [24,36]. Data are reported as OD per mg of proteins.

The extent of lipid peroxidation was determined by measuring thiobarbituric reactive substances (TBARS) using the thiobarbituric acid assay, essentially as previously described [37]. The concentration of TBARS was calculated using a molar extinction coefficient of 1.56×10^5/M/cm and expressed as nmol TBARS per g tissue.

In order to assess superoxide dismutase activity (SOD), little pieces of hippocampus were homogenized in a buffer containing 0.1 mM EDTA, 50 mM KH_2PO_4 pH 7.8, 20 mM cythocrome c, 0.1 mM xanthine, and 0.01 units of xanthine oxidase. SOD activity was measured by monitoring, spectrophotometrically (550 nm; 25 °C), the decrease in the reduction rate of cytochrome c by superoxide radicals generated by the xanthine-xanthine oxidase system. One unit of SOD activity is defined as the concentration of enzyme that inhibits cytochrome c reduction by 50% in the presence of xanthine + xanthine oxidase [38].

Glutathione reductase (GR) activity was measured in accordance with a previous report [39]. For the determination of GR activity, the decrease of NADPH absorbance at 340 nm was measured at 30 °C in homogenates from the hippocampus. The reaction mixture contained 0.2 M potassium phosphate buffer, 2 mM EDTA, 2 mM NADPH (in 10 mM Tris-HCl, pH 7), and 20 mM oxidized glutathione (GSSG). The activity was calculated using the NADPH molar extinction coefficient, 6.22×10^{-5}, considering that one unit of glutathione reductase is defined the amount of enzyme that catalyzes the reduction of 1 µmol of NADPH per minute. The specific activity is expressed in mU per g of tissue.

2.10. Immunofluorescence Analysis

Paraffin-embedded sections of hippocampus from all groups were stained with the specific monoclonal antibody against the phosphorylated form of neurofilament-M (p-NF-M,

Santa Cruz Biotechnology, Dallas, TX, USA), and slides were stained with DAPI (Sigma Aldrich, Saint Louis, MO, USA). For the analysis, images were acquired with ×40 magnification. Three random fields/section per rat were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Images were captured and visualized using a Nikon Eclipse E1000 microscope.

2.11. Statistical Analysis

Data are reported as mean values ± SEM. The program GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used to verify that raw data had a normal distribution and to perform a one-way ANOVA followed by Tukey's post-test. $p < 0.05$ was considered significant in the reported analyses.

3. Results

3.1. Glut-5 Expression, Fructose and Uric Acid Level in Hippocampus

To obtain information on the delivery of fructose to brain cells, we quantified the protein expression of Glut-5, the specific fructose transporter, as well as the levels of fructose and uric acid, one of the main products of fructose metabolism, in the hippocampus after 3 weeks of consuming a fructose-rich diet. The Glut-5 concentration was significantly higher ($p < 0.001$) in the hippocampi of F rats compared to in C rats, while this increase disappeared in FR rats (Figure 1A). In line with this result, significant increases in hippocampus levels of fructose ($p < 0.05$) and uric acid ($p < 0.05$) were found in F rats compared to C rats, while no significant differences were found in FR rats (Figure 1B,C).

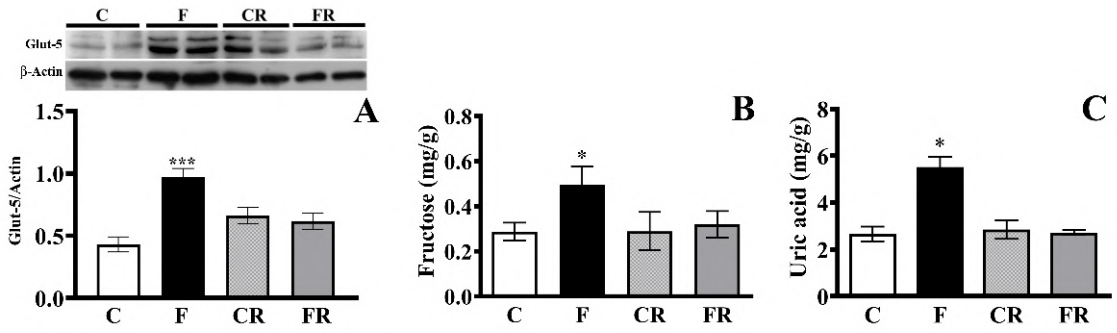

Figure 1. Hippocampus fructose handling. (**A**) Glut-5 level (with representative Western blot), (**B**) fructose and (**C**) uric acid concentrations in the hippocampi of control (C), fructose-fed (F), control rescued (CR), and fructose-rescued (FR) rats. Data are the means ± SEM of eight rats/group. * $p < 0.05$ versus control rats; *** $p < 0.001$ versus control rats. Source of variation: one-way ANOVA followed by Tukey's post-test.

3.2. Markers of Hippocampal Inflammation

We previously reported that short-term fructose feeding was associated with an increase in inflammatory markers (TNF-alpha and GFAP) in the hippocampi of young and adult rats [16]. To further clarify the brain inflammatory mechanisms activated by a fructose-rich diet and the putative ability of a subsequent 3-week control diet to rescue the fructose-induced alterations, we evaluated TNF-alpha, NF-kB activation, GFAP as marker of astrogliosis, and Iba1 as a marker of microglial activation. The TNF-alpha level was significantly higher in the hippocampi of F rats ($p < 0.01$; Figure 2A), while it returned to control levels in FR rats (Figure 2A). In agreement with results from TNF-alpha titration, in the hippocampi of F rats, we found a significant increase in the degree of NF-kB phosphorylation ($p < 0.01$), which returned to levels comparable to those of C rats in FR rats (Figure 2B). Fructose feeding was also associated with increases in Iba1 ($p < 0.01$; Figure 2C) and GFAP ($p < 0.001$; Figure 2D). After switching to the control diet, the above differences disappeared. Overall, these results show that a fructose-rich diet is associated with an

increase in key inflammation markers in the hippocampus, and this nutritional insult is fully rescued after switching to a control diet.

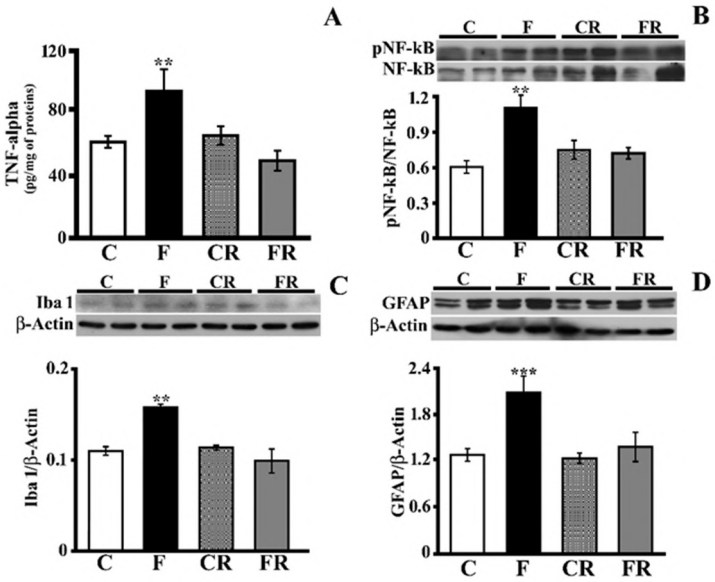

Figure 2. Inflammatory status in the hippocampus (**A**)TNF-alpha concentration (titrated by sandwich ELISA), (**B**) activation of NF-kB (expressed as the pNF-kB/NF-kB ratio with representative Western blot), (**C**) Iba1 level (with representative Western blot), (**D**) GFAP level (with representative Western blot) in protein extracts from the hippocampi of control (C), fructose-fed (F), control rescued (CR), and fructose-rescued (FR) rats. Data are the means ± SEM of eight rats/group. ** $p < 0.01$ versus control rats; *** $p < 0.001$ versus control rats. Source of variation: one-way ANOVA followed by Tukey's post-test.

Interestingly, this result was not true when the level of hippocampal Hpt, a marker of inflammation [29,40] that is very sensitive to nutritional changes [30,36], was measured (Figure 3A). Indeed, the Hpt level was greater ($p < 0.001$) in F rats with values that also remained significantly higher in FR rats compared with C rats. The increase in Hpt might represent a compensative response to a diet-induced change in Hb, as Hpt is an acute phase protein that binds to free Hb and neutralizes its pro-oxidative action, thus limiting oxidative stress [31]. This hypothesis was excluded by the finding of no alteration in the level of Hb after consumption of a fructose-rich diet (Figure 3B). Of note, the Hb level was decreased both in CR rats compared to C rats ($p < 0.01$) and in FR compared to F rats ($p < 0.05$), thus suggesting an age-dependent effect. No diet-associated variation in adiponectin, an adipokine with a neuroprotective effect [41], was detected in the hippocampi (Figure 3C).

3.3. Electron Transport Chain Pathway

Mitochondrial activity was assessed by evaluating the integrated pathway of the electron transport chain. HRR was used to determine the FCR in the hippocampus using a SUIT protocol.

As for mitochondrial oxygen consumption rates, we found a significant decrease in ADP-supported respiration with complex I- and II-linked substrates and in FCCP-stimulated respiration with complex II or complex I and II-linked substrates in F rats (Figure 4A). The analysis of PGC-1α, a marker of mitogenesis, showed no evidence of differences between the rat groups (Figure 4B).

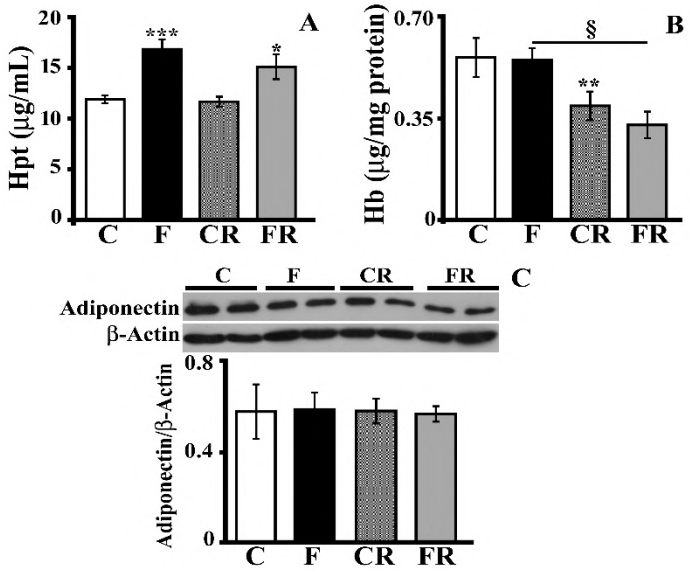

Figure 3. Haptoglobin (Hpt), hemoglobin (Hb), and adiponectin. (**A**) Hpt and (**B**) Hb levels (titrated by ELISA) and (**C**) adiponectin concentration (with representative Western blot) in protein extracts from the hippocampi of control (C), fructose-fed (F), control rescued (CR), and fructose-rescued (FR) rats. Data are the means ± SEM of eight rats/group. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$ versus control rats; § $p < 0.05$ versus fructose rats. Source of variation: one-way ANOVA followed by Tukey's post-test.

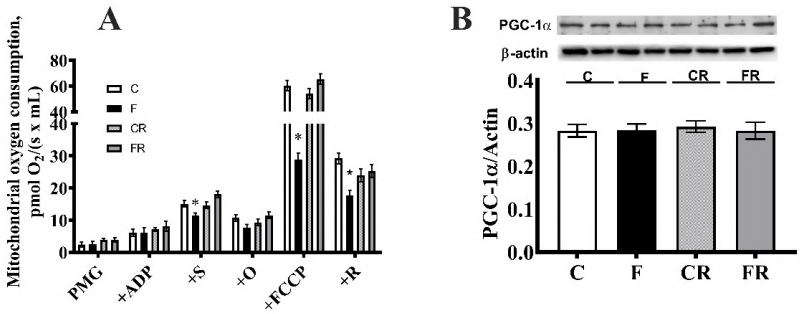

Figure 4. Mitochondrial function. (**A**) Non-normalized respiration after the addition of pyruvate + malate+ glutamate (PMG), ADP, succinate (S), oligomycin (O), FCCP, and rotenone (R), (**B**) PGC-1alpha concentration (with representative Western blot) in the hippocampi from control (C), fructose-fed (F), control-rescued (CR), and fructose-rescued (FR) rats. Values are the means ± SEM of eigth different rats. * $p < 0.05$, compared to C rats (One-way ANOVA followed by Tukey's post-test). Data are the means ± SEM of eight different rats. * $p < 0.05$ compared to C rats. Source of variation: one-way ANOVA followed by Tukey's post-test. FCCP: carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

The FCRLI, FCRPI, and coupling efficiency significantly increased in F rats (Figure 5A,C,E), while the excess capacity was significantly lower (Figure 5F). In addition, the FCF was significantly higher for complex I, while the FCF for complex II was significantly lower (Figure 5G,H). All of the above changes were completely reversed in FR rats.

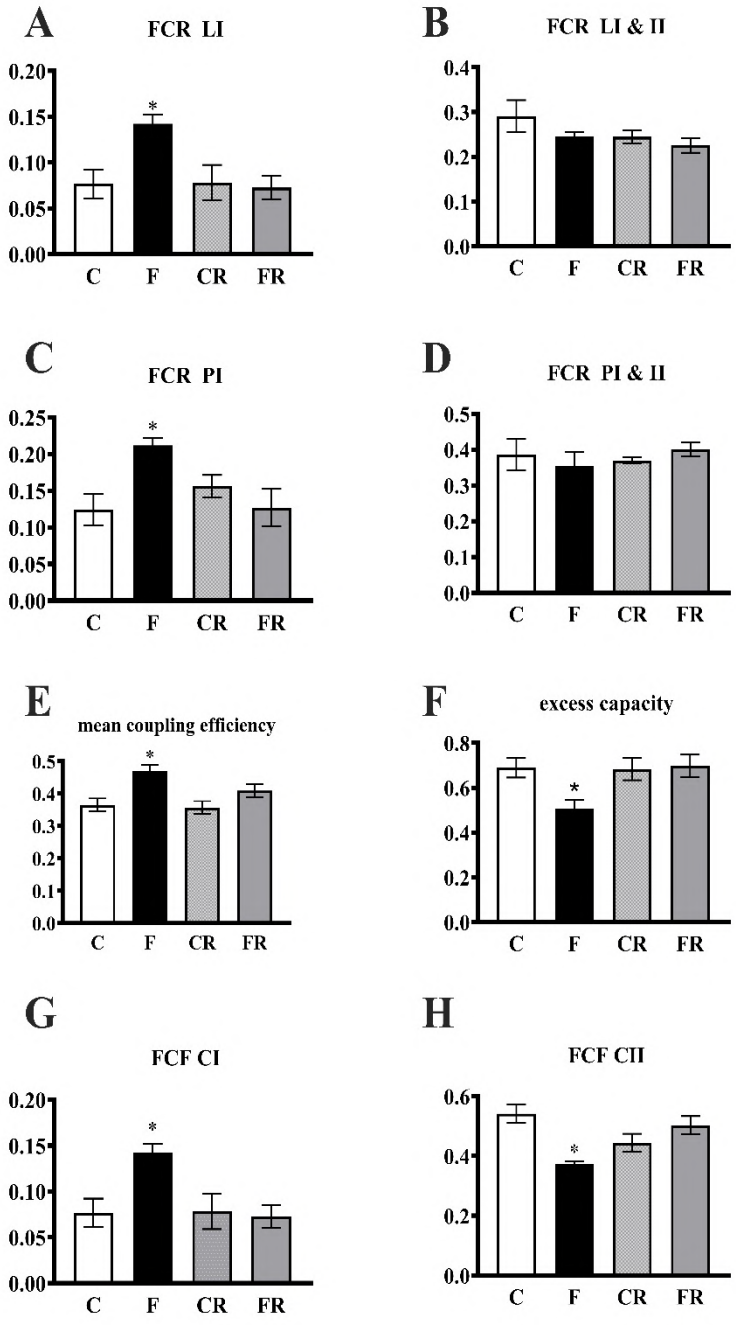

Figure 5. Integrated pathway of the electron transport chain in the hippocampus. Leak respiration with electron provision from complex I (FCR LI) (CIL)/(CI&IIE) (**A**) and complexes I and II (FCR L CI&II (CI&IIL/CI&IIE) (**B**), phosphorylating respiration with electron provision from complex I (FCR PI) (CIP/CI&IIE) (**C**) and complexes I and II (FCR PI&II) (CI&IIP/CI&IIE) (**D**), coupling efficiency of oxidative phosphorylation (1—CI&IIL/CI&IIP) (**E**), apparent excess capacity of the electron transport chain (1—CI&IIP/CI&IIE) (**F**), flux control factor (FCF) for complex I (**G**) and complex II (**H**) in the hippocampi from control (C), fructose-fed (F), control-rescued (CR), and fructose-rescued (FR) rats. Values are the means ± SEM of eight different rats. * $p < 0.05$ compared to C rats. Source of variation: one-way ANOVA followed by Tukey's post-test. CIL = leak respiration with complex I substrate; CI&IIL = leak respiration with complex I and II substrates; CIP = phosphorylating respiration with complex I substrate; CI&IIP = phosphorylating respiration with complex I and II substrates; CI&IIE = maximum capacity of the electron transport chain with complex I and II substrates.

3.4. Markers of Oxidative Status

As the strong link between mitochondrial function and reactive oxygen species (ROS) formation is well-known, we investigated the hippocampal oxidative status in the different rat groups and determined whether switching to a control diet can restore redox homeostasis alterations back to physiological levels. Lipid peroxidation was evaluated as a marker of oxidative damage to lipids, and N-Tyr, the footprint of protein oxidative damage induced by peroxynitrite [42], was assessed as a marker of oxidative damage to proteins. Further, the activity of two antioxidant enzymes, namely SOD, a frontline antioxidant enzyme catalyzing superoxide breakdown, and GSR, implicated in the recycling of reduced glutathione (GSH), was measured [43]. Enhanced diet-associated oxidative damage to lipids and proteins, namely increases in TBARS ($p < 0.05$; Figure 6A) and N-Tyr ($p < 0.01$; Figure 6B), were found in F rats. Of note, while the concentration of N-Tyr remained higher in FR with levels comparable to those found in hippocampi of F rats, the TBARS levels in FR rats returned to levels comparable to those in C rats. Likewise, we found diet-related decreases in SOD (Figure 6C) and GSR (Figure 6D) that were restored by switching back to the control diet.

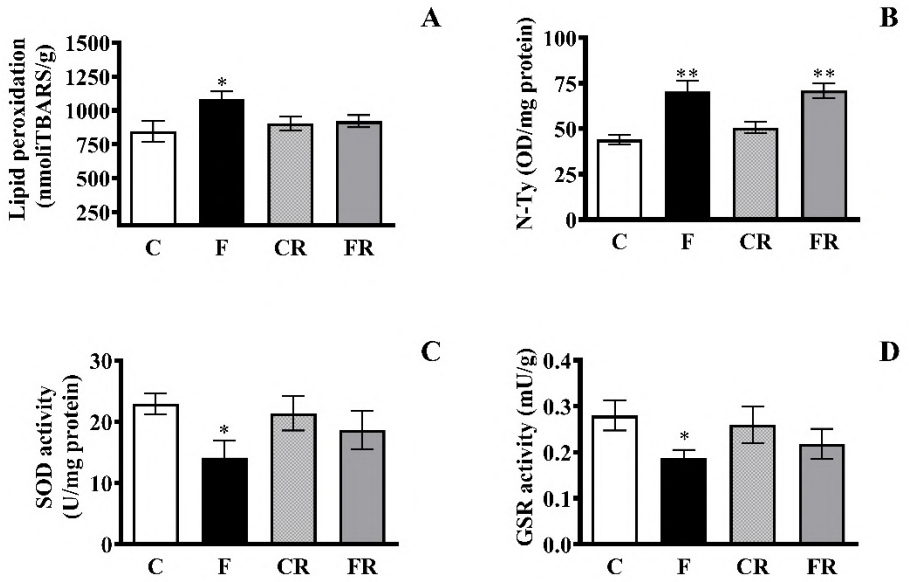

Figure 6. Markers of oxidative status. (**A**) TBARS (**B**) N-Tyr levels, (**C**) SOD activity, (**D**), GSR activity in the hippocampi of control (C), fructose-fed (F), control rescued (CR), and fructose-rescued (FR) rats. Data are the means ± SEM of eight rats/group. * $p < 0.05$ versus control rats; ** $p < 0.01$ versus control rats. Source of variation: one-way ANOVA followed by Tukey's post-test.

3.5. Analysis of Neurofilament M and Synaptic Proteins

The impacts of both the fructose-rich diet and switching to a control diet on neurofilament M were then evaluated. Neurofilaments play crucial roles in axonal transport, and their alteration has been linked to the pathogenesis of neurological disorders involving cognitive dysfunction [44]. Therefore, to further study the effect of fructose on brain functioning, the level of p-NF-M in the hippocampus was determined by immunofluorescence analysis, and a significant decrease was found in F rats, but this was restored by the rescue treatment (Figure 7A,B).

Further, the levels of key pre (synaptophysin and synaptotagmin I) and post (PSD-95) synaptic proteins were measured in the hippocampi of all groups of rats (Figure 7C–E). Synaptophysin, the most abundant presynaptic vescicle protein, plays critical dual roles in both exocytosis and exoendocytosis coupling processes [45]. Synaptotagmin I, a major calcium sensor for transmitter release, participates in the clamping of synaptic vesicle fusion in mammalian neurons [46]. The fructose-rich diet did not affect the synaptophysin

and synaptotagmin levels, while the concentration of PSD-95, which is involved in the function of neurotransmitter receptors, was significantly decreased in F rats ($p < 0.001$), but its levels returned to control values in FR rats.

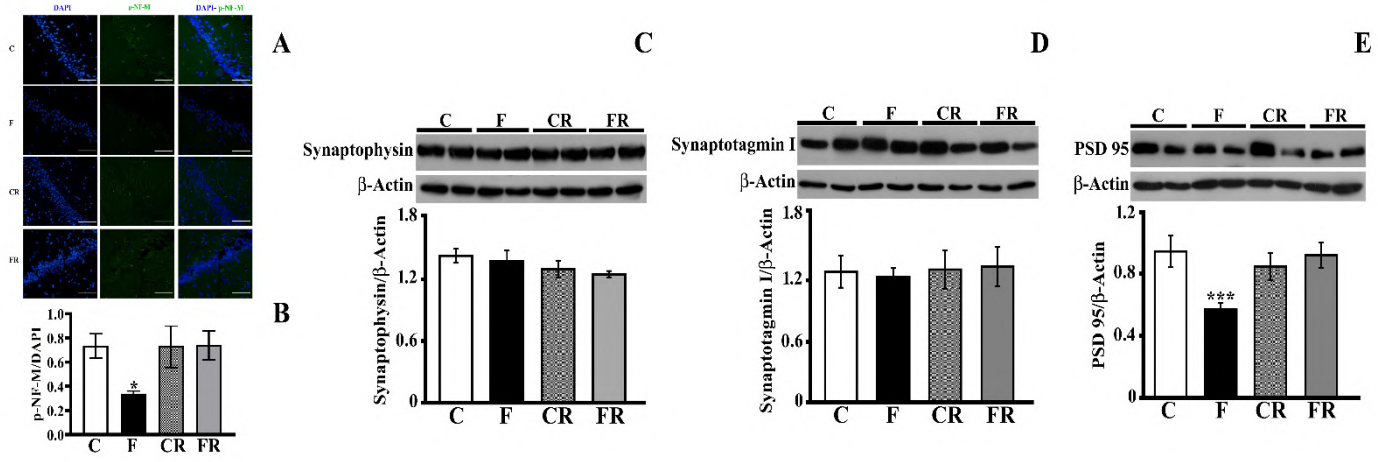

Figure 7. Markers of neuronal functioning. (**A,B**) p-Neurofilament M immunofluorescence (**C**) synapthophysin level (with representative Western blot), (**D**) synaptotagmin I (with representative Western blot), (**E**) PSD 95 level (with representative Western blot) in the hippocampi of control (C), fructose-fed (F), control rescued (CR), and fructose-rescued (FR) rats. Data are the means ± SEM of eight rats/group. * $p < 0.05$ versus control rats; *** $p < 0.001$ versus control rats. Source of variation: one-way ANOVA followed by Tukey post-test.

4. Discussion

Changes in dietary lifestyle, such as the dramatic abuse of processed foods (bakeries, snacks, breakfast cereals) and bottled fruit juices rich in HFCS, particularly among young people, may have deleterious impacts on the body as well as on brain health by disrupting neuronal metabolism and function [7]. Since most fructose is known to be metabolized by the gut and liver before reaching the systemic circulation [47], a critical issue is determining how the intake of fructose can induce changes in brain structure and function. Indeed, although in small amounts, the fructose circulating in the blood can reach the brain, as demonstrated in vivo with mice that received an oral gavage of labeled fructose [47]. Therefore, it cannot be excluded that low concentrations of dietary fructose could have a direct impact on the brain. It is also possible that the systemic metabolism of fructose might promote the release of inflammatory cytokines and/or other plasma metabolites which, when imported into the brain, could affect its metabolism and function.

The present study deals with themes of primary importance that have not yet been deeply investigated: (i) the effect of short-term fructose intake on hippocampus function in the juvenile phase, which is critical for brain development and function, and (ii) the putative reversibility of hippocampus alterations induced by this sugar by switching to a control diet. In particular, in this study, we extended our previous results [16]. Firstly, we assessed the hippocampus levels of Glut-5, fructose, and uric acid and obtained further insight into the mitochondrial compartment by carrying out a full functional analysis of the oxidative phosphorylation system. In addition, we integrated information on the oxidative status by assessing antioxidant enzyme concentrations and deepened the analysis of the inflammatory status in response to fructose. Finally, to the best of our knowledge, this is the first investigation that has aimed to analyze the possible recovery from fructose-induced metabolic modifications in the hippocampus after a short-term dietary treatment.

Several studies have revealed that dietary fructose can increase brain expression of the fructose transporter Glut-5 [17,48] and sugar metabolism [49–51]. In this study, we showed that a short-term fructose-rich diet is associated with increases in both hippocampal Glut-5 and fructose levels. Interestingly, the increased levels of uric acid found in fructose-fed rats are suggestive of an enhancement in fructose metabolism in the hippocampus. In

fact, in several tissues, it has been demonstrated that when fructose reaches the cells, fructokinase C converts it to fructose-1-phosphate with consequent decreases in intracellular phosphate and ATP levels. In turn, the low level of intracellular phosphate activates adenosine monophosphate (AMP) deaminase, with consequent degradation of AMP to inosine monophosphate and, eventually, uric acid. The consumption of AMP caused by the activation of AMP deaminase-2 reduces the cell's ability to restore ATP levels and further stimulates uric acid production [8,52,53]. Uric acid, while being an anti-oxidant in the extracellular environment, has proinflammatory activity in the intracellular environment and can induce NF-kB activation and oxidative stress [54–56]. In particular, it has been reported that uric acid causes hippocampal inflammation via the TLR4/NF-kB pathway, resulting in cognitive dysfunction [56]. As a matter of fact, an increase in NF-kB activation was found in the hippocampi of fructose-fed rats together with an increase in the key inflammatory cytokine TNF-alpha as well as glial and microglial activation, evidenced by enhanced levels of both GFAP and Iba1. It is noteworthy that the diet-induced increases in fructose and uric acid levels occurred in parallel with the hippocampal inflammatory status, since the switch to a control diet normalized brain fructose and the uric acid level and brought back almost all the inflammatory parameters to values comparable to those of control rats. This result is different from that recently reported by Fierros-Campuzano et al. (2020) [18], who described the persistence of hippocampus inflammation markers, namely the increases of IL-1β and GFAP, in a group of rats exposed to a fructose-free period after fructose intake. Nevertheless, this difference could be ascribed to the much longer duration of the fructose diet (twelve weeks).

An intriguing finding of our analysis is the increase in Hpt in fructose-fed rats in the presence of no significant change in adiponectin. The Hpt increase persisted when the rats were switched back to a control diet for further 3 weeks. We previously showed that this acute-phase protein, which is well-known for its antioxidant activity [31,57–59], is highly sensitive to nutritional insults in the brain as well as in the systemic circulation [30,36,60], and its change, which persisted even after switching back to a control diet, might represent a protective mechanism against the enhanced oxidative stress found in the hippocampus. An interesting hypothesis that certainly deserves further investigation is that plasma Hpt, which is increased by fructose intake [61], might cross the blood–brain barrier by binding to specific receptors, or it might be produced locally in the brain following microglial activation and then characterized by a slow turnover. The latter hypothesis is supported by an investigation in which Hpt was found among the major selective transcripts expressed by microglia in the hippocampi of mice injected with a cocktail of cytokines (TNF-alpha, IL-12, and IL-1β) [62].

The onset of inflammation has been frequently associated with mitochondrial dysfunction and oxidative stress [43,63]. We, therefore, sought to investigate mitochondrial respiratory function by using, for the first time in hippocampi of young rats after fructose intake, the HRR on hippocampus homogenates to maintain mitochondria in a cellular context [24].

The decrease in ADP-supported respiration that was evident only after the addition of succinate is indicative of the fact that the impairment specifically affects the function of complex II. Lower ADP-supported respiration with complex I- and II-linked substrates may result from damage to complex II, complex III, complex IV, dicarboxylate carrier, and/or the phosphorylation reactions (Adenine Nucleotide Translocator, ATP synthase and phosphate carrier). The decreased respiration measured under uncoupled conditions allows us to exclude the occurrence of an impairment in phosphorylating reactions that do not exert control over respiration in this condition. In addition, the fact that a decrease in uncoupled respiration was also evident after the addition of rotenone, a specific inhibitor of the flux from complex I to complex II, thus allowing us to measure only the flux through the respiratory chain from complex II onwards, confirms that the fructose-induced impairment is located from complex II onwards. Similar to our findings, Agrawal et al. [64] found a decrease in hippocampal mitochondrial activity using succinate, a complex II-

linked substrate after 7 weeks of consuming 15% fructose in drinking water. It has been suggested that complex II plays a role in reactive oxygen species (ROS) production under physiological and pathophysiological conditions, and defective functioning of complex II has been associated with neurodegeneration. In fact, the administration of an irreversible inhibitor of succinate dehydrogenase simulates the neuropathological and clinical features of Huntington disease (HD) in nonhuman primates [65] and evidence of the malfunctioning of complex II has been shown in patients with HD [66].

Moreover, the hippocampal mitochondria showed an increased coupling efficiency, which, in conjunction with the impairment in complex II, may contribute to the increased oxidative stress observed in F rats. In fact, uncoupling is a major mechanism in the control of mitochondrial ROS production, since it reduces the supply of electrons to the respiratory complexes and their possible interaction with oxygen [67,68]. The alteration in mitochondrial functioning is not linked to a lower organelle mass, since the hippocampus expression of PGC-1α was not altered in fructose-fed rats. The existing link between increased fructose delivery to the brain and the following mitochondrial impairment is supported by full reversal of the above changes in hippocampal mitochondria after switching back to the control diet.

ROS levels depend on the production of superoxide and its toxic metabolites as well as on the antioxidant defense mechanisms [43]. In line with the finding of mitochondria dysfunction, our results demonstrate that a fructose diet is associated with brain oxidative stress in terms of increased oxidative damage and decreased antioxidant defenses. N-Tyr and TBARS levels were significantly higher in the hippocampi of fructose-fed rats, and after switching to the control diet, TBARS returned to values comparable to those of control rats, while N-Tyr levels remained higher. This result can be explained by the fact that N-Tyr is a very stable marker of oxidative/nitrative stress [69,70] and suggests that protein turnover may control the return of the N-Tyr concentration to the initial values. As a matter of fact, brain protein turnover depends on multiple factors, such as the cell type, intracellular environment, specific protein functions, and protein interactions [71], with the half-lives of neuronal protein ranging from <2 to >14 days [71].

The unbalanced redox homeostasis is corroborated by the decrease in the activity of two antioxidant enzymes: SOD and GSR [43]. Consistent with the importance of SOD and GSR for cellular health, many human diseases of the central nervous system involve perturbations in these enzymes [72,73]. Regarding the analysis of these antioxidant enzymes, 3 weeks after the cessation of the fructose-rich diet, their activities returned to control values, suggesting that the fructose-induced alterations in redox homeostasis in young age can be reversed by a diet correction for an equal period of time.

To further highlight the key neuronal components influenced by fructose intake, we studied NF-M, as it is involved in the stabilization of newly-sprouted axonal processes [74]. Neurofilaments guarantee the morphology of neurons and are crucial for axonal transport [74–76]. It is worth mentioning that disruption of the cytoskeletal framework of neurons typically triggers dystrophic neurites, thus representing a key feature of neurodegenerative diseases [77–79]. Based on the results of the immunofluorescence analysis, it can be suggested that alterations in NFs following fructose intake might give rise to dysfunction in axonal transport. This alteration seems to be reversible by switching to a control diet. A similar result was obtained by assessing the amount of synaptic proteins in the hippocampi of the rats in the different groups. In fact, a significant decrease in the post-synaptic critical protein PSD-95 was found in the hippocampi of fructose-fed rats, which was recovered by interrupting the fructose diet and switching to a control diet.

5. Conclusions

The picture that emerges from this study, which was conducted on a young rodent model, confirms that fructose can strongly impact brain function in juvenile age by promoting hippocampal inflammation, mitochondrial dysfunction, oxidative stress, alteration in cytoskeletal components, and post-synaptic proteins. These changes could undoubtedly

have an important impact on neuronal activity and, in general, on cognitive function, especially in the youth, a very critical phase of brain development. Most of the alterations induced by a fructose-rich diet can be rescued by switching back to a control diet. Notable exceptions are represented by Hpt and N-Tyr, markers of inflammation and oxidative stress, respectively, which remain higher as an imprint of the previous damage. Investigation of the real consequences of the persistent alterations in these markers certainly deserves further attention and may represent an issue for further study. It cannot be excluded that a longer period of fructose intake could promote cerebral alterations to a greater extent that are difficult to revert with the return to a healthy diet. This study, once again, draws attention to the need to foresee the use of alternative sugars to HFCS with less dangerous effects to preserve the brain health of young populations.

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Institutional Review Board Statement: The study was conducted according to the guidelines set by the Italian Health Ministry. All experimental procedures involving animals were approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II (448/2019-PR).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

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References

1. Bray, G.A.; Nielsen, S.J.; Popkin, B.M. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. *Am. J. Clin. Nutr.* **2004**, *79*, 537–543. [CrossRef] [PubMed]
2. Bray, G.A. Soft drink consumption and obesity: It is all about fructose. *Curr. Opin. Lipidol.* **2010**, *21*, 51–57. [CrossRef] [PubMed]
3. Schulze, M.B.; Manson, J.E.; Ludwig, D.S.; Colditz, G.A.; Stampfer, M.J.; Willett, W.C.; Hu, F.B. Sugar-sweetened beverages, weight gain, and incidence of type 2 diabetes in young and middle-aged women. *JAMA* **2004**, *292*, 927–934. [CrossRef]
4. Livesey, G.; Taylor, R. Fructose consumption and consequences for glycation, plasma triacylglycerol, and body weight: Metaanalyses and meta-regression models of intervention studies. *Am. J. Clin. Nutr.* **2008**, *88*, 1419–1437. [CrossRef] [PubMed]
5. Alwahsh, S.M.; Gebhardt, R. Dietary fructose as a risk factor for non-alcoholic fatty liver disease (NAFLD). *Arch. Toxicol.* **2017**, *91*, 1545–1563. [CrossRef]
6. Mastrocola, R.; Collino, M.; Rogazzo, M.; Medana, C.; Nigro, D.; Boccuzzi, G.; Aragno, M. Advanced glycation end products promote hepatosteatosis by interfering with SCAP-SREBP pathway in fructose-drinking mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2013**, *305*, G398–G407. [CrossRef] [PubMed]
7. Spagnuolo, M.S.; Iossa, S.; Cigliano, L. Sweet but Bitter: Focus on Fructose Impact on Brain Function in Rodent Models. *Nutrients* **2020**, *13*, 1. [CrossRef]
8. Johnson, R.J.; Gomez-Pinilla, F.; Nagel, M.; Nakagawa, T.; Rodriguez-Iturbe, B.; Sanchez-Lozada, L.G.; Tolan, D.R.; Lanaspa, M.A. Cerebral Fructose metabolism as a Potential Mechanism Driving Alzheimer's Disease. *Front. Aging Neurosci.* **2020**, *12*, 560865. [CrossRef] [PubMed]
9. Yin, Q.; Ma, Y.; Hong, Y.; Hou, X.; Chen, J.; Shen, C.; Sun, M.; Shang, Y.; Dong, S.; Zeng, Z.; et al. Lycopene attenuates insulin signaling deficits, oxidative stress, neuroinflammation, and cognitive impairment in fructose-drinking insulin resistant rats. *Neuropharmacology* **2014**, *86*, 389–396. [CrossRef] [PubMed]
10. Van der Borght, K.; Köhnke, R.; Göransson, N.; Deierborg, T.; Brundin, P.; Erlanson-Albertsson, C.; Lindqvist, A. Reduced Neurogenesis in the Rat Hippocampus Following High Fructose Consumption. *Regul. Pept.* **2011**, *167*, 26–30. [CrossRef]
11. Mastrocola, R.; Nigro, D.; Cento, A.S.; Chiazza, F.; Collino, M.; Aragno, M. High-fructose intake as risk factor for neurodegeneration: Key role for carboxy methyllysine accumulation in mice hippocampal neurons. *Neurobiol. Dis.* **2016**, *89*, 65–75. [CrossRef]

12. Li, J.M.; Ge, C.X.; Xu, M.X.; Wang, W.; Yu, R.; Fan, C.Y.; Kong, L.D. Betaine recovers hypothalamic neural injury by inhibiting astrogliosis and inflammation in fructose-fed rats. *Mol. Nutr. Food Res.* **2015**, *59*, 189–202. [CrossRef]
13. Wu, H.W.; Ren, L.F.; Zhou, X.; Han, D.W. A high-fructose diet induces hippocampal insulin resistance and exacerbates memory deficits in male Sprague-Dawley rats. *Nutr. Neurosci.* **2015**, *18*, 323–328. [CrossRef]
14. Hsu, T.M.; Konanur, V.R.; Taing, L.; Usui, R.; Kayser, B.D.; Goran, M.I.; Kanoski, S.E. Effects of sucrose and high fructose corn syrup consumption on spatial memory function and hippocampal neuroinflammation in adolescent rats. *Hippocampus* **2015**, *25*, 227–239. [CrossRef] [PubMed]
15. Ross, A.P.; Bartness, T.J.; Mielke, J.G.; Parent, M.B. A high fructose diet impairs spatial memory in male rats. *Neurobiol. Learn. Mem.* **2009**, *92*, 410–416. [CrossRef] [PubMed]
16. Cigliano, L.; Spagnuolo, M.S.; Crescenzo, R.; Cancelliere, R.; Iannotta, L.; Mazzoli, A.; Liverini, G.; Iossa, S. Short-Term Fructose Feeding Induces Inflammation and Oxidative Stress in the Hippocampus of Young and Adult Rats. *Mol. Neurobiol.* **2018**, *55*, 2869–2883. [CrossRef]
17. Jiménez-Maldonado, A.; Ying, Z.; Byun, H.R.; Gomez-Pinilla, F. Short-term fructose ingestion affects the brain independently from establishment of metabolic syndrome. *Biochim. Biophys. Acta Mol. Basis Dis.* **2018**, *1864*, 24–33. [CrossRef] [PubMed]
18. Fierros-Campuzano, J.; Ballesteros-Zebadúa, P.; Manjarrez-Marmolejo, J.; Aguilera, P.; Méndez-Diaz, M.; Prospero-García, O.; Franco-Pérez, J. Irreversible hippocampal changes induced by high fructose diet in rats. *Nutr. Neurosci.* **2020**, *3*, 1–13. [CrossRef] [PubMed]
19. Ford, C.N.; Slining, M.M.; Popkin, B.M. Trends in dietary intake among US 2- to 6-year-old children, 1989–2008. *J. Acad. Nutr. Diet.* **2013**, *113*, 35–42. [CrossRef] [PubMed]
20. Hsu, T.M.; Kanoski, S.E. Blood-brain barrier disruption: Mechanistic links between Western diet consumption and dementia. *Front. Aging Neurosci.* **2014**, *6*, 88. [CrossRef]
21. Clark, K.A.; Alves, J.M.; Jones, S.; Yunker, A.G.; Luo, S.; Cabeen, R.P.; Angelo, B.; Xiang, A.H.; Page, K.A. Dietary Fructose Intake and Hippocampal Structure and Connectivity during Childhood. *Nutrients* **2020**, *12*, 909. [CrossRef] [PubMed]
22. Davidson, T.L.; Chan, K.; Jarrard, L.E.; Kanoski, S.E.; Clegg, D.J.; Benoit, S.C. Contributions of the Hippocampus and Medial Prefrontal Cortex to Energy and BodyWeight Regulation. *Hippocampus* **2009**, *19*, 235–252. [CrossRef] [PubMed]
23. Hannapel, R.C.; Henderson, Y.H.; Nalloor, R.; Vazdarjanova, A.; Parent, M.B. Ventral hippocampal neurons inhibit postprandial energy intake. *Hippocampus* **2017**, *27*, 274–284. [CrossRef]
24. Crescenzo, R.; Spagnuolo, M.S.; Cancelliere, R.; Iannotta, L.; Mazzoli, A.; Gatto, C.; Iossa, S.; Cigliano, L. Effect of Initial Aging and High-Fat/High-Fructose Diet on Mitochondrial Bioenergetics and Oxidative Status in Rat Brain. *Mol. Neurobiol.* **2019**, *56*, 7651–7663. [CrossRef] [PubMed]
25. Spagnuolo, M.S.; Bergamo, P.; Crescenzo, R.; Iannotta, L.; Treppiccione, L.; Iossa, S.; Cigliano, L. Brain Nrf2 pathway, autophagy, and synaptic function proteins are modulated by a short-term fructose feeding in young and adult rats. *Nutr. Neurosci.* **2020**, *23*, 309–320. [CrossRef] [PubMed]
26. Spagnuolo, M.S.; Pallottini, V.; Mazzoli, A.; Iannotta, L.; Tonini, C.; Morone, B.; Ståhlman, M.; Crescenzo, R.; Strazzullo, M.; Iossa, S.; et al. A Short-Term Western Diet Impairs Cholesterol Homeostasis and Key Players of Beta Amyloid Metabolism in Brain of Middle-Aged Rats. *Mol. Nutr. Food Res.* **2020**, *64*, e2000541. [CrossRef]
27. Spagnuolo, M.S.; Maresca, B.; Mollica, M.P.; Cavaliere, G.; Cefaliello, C.; Trinchese, G.; Esposito, M.G.; Scudiero, R.; Crispino, M.; Abrescia, P.; et al. Haptoglobin increases with age in rat hippocampus and modulates Apolipoprotein E mediated cholesterol trafficking in neuroblastoma cell lines. *Front. Cell Neurosci.* **2014**, *8*, 212. [CrossRef]
28. Spagnuolo, M.S.; Donizetti, A.; Iannotta, L.; Aliperti, V.; Cupidi, C.; Bruni, A.C.; Cigliano, L. Brain-derived neurotrophic factor modulates cholesterol homeostasis and Apolipoprotein E synthesis in human cell models of astrocytes and neurons. *J. Cell Physiol.* **2018**, *233*, 6925–6943. [CrossRef]
29. Spagnuolo, M.S.; Maresca, B.; La Marca, V.; Carrizzo, A.; Veronesi, C.; Cupidi, C.; Piccoli, T.; Maletta, R.G.; Bruni, A.C.; Abrescia, P.; et al. Haptoglobin interacts with apolipoprotein E and beta-amyloid and influences their crosstalk. *ACS Chem. Neurosci.* **2014**, *5*, 837–847. [CrossRef]
30. Mazzoli, A.; Spagnuolo, M.S.; Gatto, C.; Nazzaro, M.; Cancelliere, R.; Crescenzo, R.; Iossa, S.; Cigliano, L. Adipose Tissue and Brain Metabolic Responses to Western Diet-Is There a Similarity between the Two? *Int. J. Mol. Sci.* **2020**, *21*, 786. [CrossRef]
31. Spagnuolo, M.S.; Cigliano, L.; Abrescia, P. The binding of haptoglobin to apolipoprotein AI: Influence of hemoglobin and concanavalin A. *Biol. Chem.* **2003**, *384*, 1593–1596. [CrossRef] [PubMed]
32. Burtscher, J.; Zangrandi, L.; Schwarzer, C.; Gnaiger, E. Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues revealed by high- resolution respirometry. *Mitochondrion* **2015**, *25*, 104–112. [CrossRef]
33. Pesta, D.; Gnaiger, E. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods Mol. Biol.* **2012**, *810*, 25–58. [CrossRef] [PubMed]
34. Gnaiger, E. Capacity of oxidative phosphorylation in human skeletal muscle: New perspectives of mitochondrial physiology. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 1837–1845. [CrossRef]
35. Gnaiger, E. *Mitochondrial Pathways and Respiratory Control. An Introduction to OXPHOS Analysis. IOROBOROS MiPNet Publications*, 4th ed.; Steiger Druck GmbH: Axams, Austria, 2014; ISBN 978-973-9502399-9502398-9502390.
36. Spagnuolo, M.S.; Mollica, M.P.; Maresca, B.; Cavaliere, G.; Cefaliello, C.; Trinchese, G.; Scudiero, R.; Crispino, M.; Cigliano, L. High fat diet and inflammation—Modulation of haptoglobin level in rat brain. *Front. Cell Neurosci.* **2015**, *9*, 479. [CrossRef]

37. Crescenzo, R.; Mazzoli, A.; Cancelliere, R.; Bianco, F.; Giacco, A.; Liverini, G.; Dulloo, A.G.; Iossa, S. Polyunsaturated Fatty Acids Stimulate De novo Lipogenesis and Improve Glucose Homeostasis during Refeeding with High Fat Diet. *Front. Physiol.* **2017**, *8*, 178. [CrossRef]
38. Flohè, L.; Otting, F. Superoxide dismutase assay. *Meth. Enzymol.* **1974**, *105*, 93–104.
39. Carlberg, I.; Mannervik, B. Glutathione Reductase. *Meth. Enzymol.* **1958**, *113*, 484–485.
40. Cigliano, L.; Spagnuolo, M.S.; Abrescia, P. Quantitative variations of the isoforms in haptoglobin 1-2 and 2-2 individual phenotypes. *Arch. Biochem. Biophys.* **2003**, *416*, 227–237. [CrossRef]
41. Bloemer, J.; Pinky, P.D.; Govindarajulu, M.; Hong, H.; Judd, R.; Amin, R.H.; Moore, T.; Dhanasekaran, M.; Reed, M.N.; Suppiramaniam, V. Role of Adiponectin in Central Nervous System Disorders. *Neural Plast.* **2018**, *2018*, 4593530. [CrossRef]
42. Halliwell, B.; Gutteridge, J.M.C. Free Radicals and Antioxidants in the Year 2000: A Historical Look to the Future. *Ann. N. Y. Acad. Sci.* **2000**, *899*, 136–147. [CrossRef]
43. Goodfellow, M.J.; Borcar, A.; Proctor, J.L.; Greco, T.; Rosenthal, R.E.; Fiskum, G. Transcriptional activation of antioxidant gene expression by Nrf2 protects against mitochondrial dysfunction and neuronal death associated with acute and chronic neurodegeneration. *Exp. Neurol.* **2020**, *328*, 113247. [CrossRef]
44. Lee, J.C.; Park, J.H.; Ahn, J.H.; Park, J.; Kim, I.H.; Cho, J.H.; Shin, B.N.; Lee, T.K.; Kim, H.; Song, M.; et al. Effects of chronic scopolamine treatment on cognitive impairment and neurofilament expression in the mouse hippocampus. *Mol. Med. Rep.* **2018**, *17*, 1625–1632. [CrossRef]
45. Xie, S.; Girshick, R.; Dollar, P.; Tu, Z.; He, K. Aggregated Residual Transformations for Deep Neural Networks. *arXiv* **2017**, arXiv:1611.05431v2, 1492–1500.
46. Courtney, N.A.; Bao, H.; Briguglio, J.S.; Chapman, E.R. Synaptotagmin 1 clamps synaptic vescicle fusion in mammalian neurons independent of clompexin. *Nat. Commun.* **2019**, *10*, 4076. [CrossRef]
47. Jang, C.; Hui, S.; Lu, W.; Cowan, A.J.; Morscher, R.J.; Lee, G.; Liu, W.; Tesz, G.J.; Birnbaum, M.J.; Rabinowitz, J.D. The Small Intestine Converts Dietary Fructose into Glucose and Organic Acids. *Cell Metab.* **2018**, *27*, 351–361.e3. [CrossRef]
48. Shu, H.J.; Isenberg, K.; Cormier, R.J.; Benz, A.; Zorumski, C.F. Expression of fructose sensitive glucose transporter in the brains of fructose-fed rats. *Neuroscience* **2006**, *140*, 889–895. [CrossRef]
49. Oppelt, S.A.; Zhang, W.; Tolan, D.R. Specific regions of the brain are capable of fructose metabolism. *Brain Res.* **2017**, *1657*, 312–322. [CrossRef]
50. Meakin, P.J.; Fowler, M.J.; Rathbone, A.J.; Allen, L.M.; Ransom, B.R.; Ray, D.E.; Brown, A.M. Fructose metabolism in the adult mouse optic nerve, a central white matter tract. *J. Cereb. Blood Flow Metab.* **2007**, *27*, 86–99. [CrossRef]
51. Wu, K.L.; Hung, C.Y.; Chan, J.Y.; Wu, C.-W. An increase in adenosine-5′-triphosphate (ATP) content in rostral ventrolateral medulla is engaged in the high fructose diet-induced hypertension. *J. Biomed. Sci.* **2014**, *21*, 8. [CrossRef] [PubMed]
52. Lanaspa, M.A.; Cicerchi, C.; Garcia, G.; Li, N.; Roncal-Jimenez, C.A.; Rivard, C.J.; Hunter, B.; Andrés-Hernando, A.; Ishimoto, T.; Sanchez-Lozada, L.G.; et al. Counteracting roles of AMP deaminase and AMP kinase in the development of fatty liver. *PLoS ONE* **2012**, *7*, e48801. [CrossRef]
53. Cicerchi, C.; Li, N.; Kratzer, J.; Garcia, G.; Roncal-Jimenez, C.A.; Tanabe, K.; Hunter, B.; Rivard, C.J.; Sautin, Y.Y.; Gaucher, E.A.; et al. Uric acid-dependent inhibition of AMP kinase induces hepatic glucose production in diabetes and starvation: Evolutionary implications of the uricase loss in hominids. *FASEB J.* **2014**, *28*, 3339–3350. [CrossRef]
54. Johnson, R.J.; Nakagawa, T.; Sanchez-Lozada, L.G.; Shafiu, M.; Sundaram, S.; Le, M.; Lanaspa, M.A. Sugar, uric acid and the etiology of diabetes and obesity. *Diabetes* **2013**, *62*, 3307–3315. [CrossRef]
55. Shao, X.; Gu, H.; Wang, Z.; Chai, X.; Tian, Y.; Shi, G. Highly selective electrochemical strategy for monitoring of cerebral Cu2+ based on a carbon Dot-TPEA hybridized surface. *Anal. Chem.* **2013**, *85*, 418–425. [CrossRef]
56. Shao, X.; Lu, W.; Gao, F.; Li, D.; Hu, J.; Li, Y.; Zuo, Z.; Jie, H.; Zhao, Y.; Cen, X. Uric Acid Induces Cognitive Dysfunction through Hippocampal Inflammation in Rodents and Humans. *J. Neurosci.* **2016**, *36*, 10990–11005. [CrossRef]
57. Salvatore, A.; Cigliano, L.; Bucci, E.M.; Corpillo, D.; Velasco, S.; Carlucci, A.; Pedone, C.; Abrescia, P. Haptoglobin binding to apolipoprotein A-I prevents damage from hydroxyl radicals on its stimulatory activity of the enzyme lecithin-cholesterol acyl-transferase. *Biochemistry* **2007**, *46*, 11158–11168. [CrossRef]
58. Maresca, B.; Spagnuolo, M.S.; Cigliano, L. Haptoglobin modulates beta-amyloid uptake by U-87 MG astrocyte cell line. *J. Mol. Neurosci.* **2015**, *56*, 35–47. [CrossRef] [PubMed]
59. Spagnuolo, M.S.; Cigliano, L.; Maresca, B.; Pugliese, C.R.; Abrescia, P. Identification of plasma haptoglobin forms which loosely bind hemoglobin. *Biol. Chem.* **2011**, *392*, 371–376. [CrossRef] [PubMed]
60. Cigliano, L.; Pugliese, C.R.; Spagnuolo, M.S.; Palumbo, R.; Abrescia, P. Haptoglobin binds the antiatherogenic protein apolipoprotein E—Impairment of apolipoprotein E stimulation of both lecithin:cholesterol acyltransferase activity and cholesterol uptake by hepatocytes. *FEBS J.* **2009**, *276*, 6158–6171. [CrossRef] [PubMed]
61. Crescenzo, R.; Cigliano, L.; Mazzoli, A.; Cancelliere, R.; Carotenuto, R.; Tussellino, M.; Liverini, G.; Iossa, S. Early Effects of a Low Fat, Fructose-Rich Diet on Liver Metabolism, Insulin Signaling, and Oxidative Stress in Young and Adult Rats. *Front. Physiol.* **2018**, *9*, 411. [CrossRef] [PubMed]
62. Lee, D.C.; Ruiz, C.R.; Lebson, L.; Selenica, M.L.; Rizer, J.; Hunt, J.B., Jr.; Rojiani, R.; Reid, P.; Kammath, S.; Nash, K.; et al. Aging enhances classical activation but mitigates alternative activation in the central nervous system. *Neurobiol. Aging* **2013**, *34*, 1610–1620. [CrossRef]

63. Roberts, R.A.; Smith, R.A.; Safe, S.; Szabo, C.; Tjalkens, R.B.; Robertson, F.M. Toxicological and pathophysiological roles of reactive oxygen and nitrogen species. *Toxicology* **2010**, *276*, 85–94. [CrossRef]
64. Agrawal, R.; Noble, E.; Vergnes, L.; Ying, Z.; Reue, K.; Gomez-Pinilla, F. Dietary fructose aggravates the pathobiology of traumatic brain injury by influencing energy homeostasis and plasticity. *J. Cereb. Blood Flow Metab.* **2016**, *36*, 941–953. [CrossRef]
65. Brouillet, E.; Hantraye, P.; Ferrante, R.J.; Dolan, R.; Leroy-Willig, A.; Kowall, N.W.; Beal, M.F. Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7105–7109. [CrossRef] [PubMed]
66. Benchoua, A.; Trioulier, Y.; Zala, D.; Gaillard, M.C.; Lefort, N.; Dufour, N.; Saudou, F.; Elalouf, J.F.; Hirsch, E.; Hantraye, P.; et al. Involvement of Mitochondrial Complex II Defects in Neuronal Death Produced by N-Terminus Fragment of Mutated Huntingtin. *Mol. Biol. Cell* **2006**, *17*, 1652–1663. [CrossRef]
67. Tseng, Y.H.; Cypess, A.M.; Kahn, C.R. Cellular bioenergetics as a target for obesity therapy. *Nat. Rev. Drug Discov.* **2010**, *9*, 465–482. [CrossRef]
68. Skulachev, V.P. Uncoupling: New approaches to an old problem of bioenergetics. *Biochim. Biophys. Acta* **1998**, *1363*, 100–124. [CrossRef]
69. Herce-Pagliai, C.; Kotecha, S.; Shuker, D.E.G. Analytical Methods for 3-Nitrotyrosine as a Marker of Exposure to Reactive Nitrogen Species: A Review. *Nitric Oxide* **1998**, *2*, 324–336. [CrossRef]
70. Frijhoff, J.; Winyard, P.G.; Zarkovic, N.; Davies, S.S.; Stocker, R.; Cheng, D.; Knight, A.R.; Taylor, E.L.; Oettrich, J.; Ruskovska, T.; et al. Clinical Relevance of Biomarkers of Oxidative Stress. *Antioxid. Redox Signal.* **2015**, *23*, 1144–1170. [CrossRef] [PubMed]
71. Dörrbaum, A.R.; Kochen, L.; Langer, J.D.; Schuman, E.M. Local and global influences on protein turnover in neurons and glia. *Elife* **2018**, *7*, e34202. [CrossRef] [PubMed]
72. Lee, K.H.; Myeounghoon, C.; Lee, B.H. Neuroprotective Effect of Antioxidants in the Brain. *Int. J. Mol. Sci.* **2020**, *21*, 7152. [CrossRef] [PubMed]
73. Hajizadeh Moghaddam, A.; Jelodar, S.K.; Ranjbar, M. Hesperetin nanoparticles attenuate anxiogenic-like behavior and cerebral oxidative stress through the upregulation of antioxidant enzyme expression in experimental dementia of Alzheimer's type. *Neurol. Res.* **2020**, *42*, 477–486. [CrossRef]
74. Lee, M.K.; Cleveland, D.W. Neuronal intermediate filaments. *Annu. Rev. Neurosci.* **1996**, *19*, 187–217. [CrossRef] [PubMed]
75. Sánchez, I.; Hassinger, L.; Sihag, R.K.; Cleveland, D.W.; Mohan, P.; Nixon, R.A. Local control of neurofilament accumulation during radial growth of myelinating axons in vivo. Selective role of site-specific phosphorylation. *J. Cell Biol.* **2000**, *151*, 1013–1024. [CrossRef] [PubMed]
76. Yabe, J.T.; Chan, W.K.; Chylinski, T.M.; Lee, S.; Pimenta, A.F.; Shea, T.B. The predominant form in which neurofilament subunits undergo axonal transport varies during axonal initiation, elongation, and maturation. *Cell Motil. Cytoskelet.* **2001**, *48*, 61–83. [CrossRef]
77. Julien, J.P. Neurofilament functions in health and disease. *Curr. Opin. Neurobiol.* **1999**, *9*, 554–560. [CrossRef]
78. Perrot, R.; Berges, R.; Bocquet, A.; Eyer, J. Review of the multiple aspects of neurofilament functions, and their possible contribution to neurodegeneration. *Mol. Neurobiol.* **2008**, *38*, 27–65. [CrossRef]
79. Vickers, J.C.; Kirkcaldie, M.T.; Phipps, A.; King, A.E. Alterations in neurofilaments and the transformation of the cytoskeleton in axons may provide insight into the aberrant neuronal changes of Alzheimer's disease. *Brain Res. Bull.* **2016**, *126 Pt 3*, 324–333. [CrossRef]

Chapter 3

Long-Lasting Impact of Sugar Intake on Neurotrophins and Neurotransmitters from Adolescence to Young Adulthood in Rat Frontal Cortex

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Long-Lasting Impact of Sugar Intake on Neurotrophins and Neurotransmitters from Adolescence to Young Adulthood in Rat Frontal Cortex

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Abstract

The detrimental impact of fructose, a widely used sweetener in industrial foods, was previously evidenced on various brain regions. Although adolescents are among the highest consumers of sweet foods, whether brain alterations induced by the sugar intake during this age persist until young adulthood or are rescued returning to a healthy diet remains largely unexplored. To shed light on this issue, just weaned rats were fed with a fructose-rich or control diet for 3 weeks. At the end of the treatment, fructose-fed rats underwent a control diet for a further 3 weeks until young adulthood phase and compared with animals that received from the beginning the healthy control diet. We focused on the consequences induced by the sugar on the main neurotrophins and neurotransmitters in the frontal cortex, as its maturation continues until late adolescence, thus being the last brain region to achieve a full maturity. We observed that fructose intake induces inflammation and oxidative stress, alteration of mitochondrial function, and changes of brain-derived neurotrophic factor (BDNF) and neurotrophin receptors, synaptic proteins, acetylcholine, dopamine, and glutamate levels, as well as increased formation of the glycation end-products *Nε*-carboxymethyllysine (CML) and *Nε*-carboxyethyllysine (CEL). Importantly, many of these alterations (BDNF, CML, CEL, acetylcholinesterase activity, dysregulation of neurotransmitters levels) persisted after switching to the control diet, thus pointing out to the adolescence as a critical phase, in which extreme attention should be devoted to limit an excessive consumption of sweet foods that can affect brain physiology also in the long term.

Keywords Adolescent rat · Frontal cortex · Fructose diet · Brain-derived neurotrophic factor · Neurotransmitters · Mitochondria · Inflammation

Introduction

Fructose, a reducing monosaccharide present in fruit and honey, is the major component of the two most used sweeteners, namely, sucrose and high fructose corn syrup (HFCS). In the last decades, HFCS utilization has grown, essentially because of its longer shelf life, cheaper production, and higher sweetness which increases the palatability of sugary beverages, baked and processed foods [1, 2]. The rise in the intake of diets rich in sweeteners, mostly sweetened beverages (fruit juices, alcopops and sport drinks), in young and adolescents is particularly troubling [3–6]. Indeed, this kind of diet leads to a marked increase in daily fructose consumption that, compared to a natural consumption of 16–24 g/day with fruits and honey, can reach 80 g/day, which represents the 17–20% of the daily caloric intake [7–9].

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Excessive dietary sugar intake, both in early and later life, has also been associated with altered brain metabolism and behavioral functioning [10–13]. In this context, it is noteworthy that brain maturation, particularly in frontal cortex, continues approximately until early adulthood (24 years of age) [14–16], rendering this brain area particularly susceptible to developmental disruption due to early-life nutritional or environmental insults [17]. Indeed, the consumption of high-fat or high-sugar diets during adolescence was associated with both deficits in executive functioning and a reduced volume of the frontal cortical region in humans [18–21]. Therefore, the assessment of the sugar impact on brain-related outcomes is crucial, particularly during the critical windows of growth and development, when key features of brain tissue structure and function are establishing [22]. While several studies outlined the detrimental effect of fructose on hypothalamus or hippocampus [13, 23, 24], very little information is available on the impact of this sugar on frontal cortex. In this regard, we previously reported that, in both adolescent and adult rats, a short-term fructose intake affects redox homeostasis, autophagy, and the expression of synaptic markers [25]. Furthermore, long-term fructose drinking was associated with neuroinflammation, altered insulin signaling and cognitive impairment in frontal cortex [26, 27].

Whether the alterations induced by fructose intake during adolescence (postnatal day 30–60, P30–P60) are rescued returning to a healthy diet or persist until young adulthood is a critical issue that remains unexplored. Therefore, we focused on the frontal cortex of adolescent rats fed a fructose-rich (F) or control diet (C) for 3 weeks. After this period, to highlight whether the effects induced by the short-term fructose diet persist or are rescued, fructose-fed rats were fed a control diet for a further 3 weeks (FR) until young adulthood phase and compared with animals that received the control diet for the entire period (CR). Thus, we investigated putative damages induced by the sugar intake on brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and their specific receptors, which are crucial in modulating survival and development of the nervous system, synaptic function and plasticity, learning and memory [28, 29], as well as we evaluated the sugar impact on inflammation and oxidative stress. Moreover, a mass spectrometry-based metabolomic analysis was carried out to ascertain whether fructose intake also influences the homeostasis of amino acids, amino acid derivatives and other polar metabolites, particularly the main neurotransmitters, in the frontal cortex.

Materials and Methods

Materials

Bovine serum albumin fraction V (BSA; catalogue n. A6588), amino acids (catalogue n. LAA21), dopamine (catalogue n. H8502), acetylcholine (catalogue n. A6625) tyramine (catalogue n. T2879), γ-aminobutyric acid (GABA, catalogue n. 03,835), DL-lysine-4,4,5,5-d_4 dihydrochloride (*d4*-lysine, catalogue n. 489,034), formic acid (catalogue n. 5.33002), salts, and buffers were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Nε*-carboxymethyllysine (CML, catalogue n. HAA2950) and *Nε*-carboxyethyllysine (CEL, catalogue n. HAA2940) were obtained from Iris Biotech (Marktredwitz, Germany). Acetonitrile (catalogue n. 1.00029), water (catalogue n. 1.15333), and ammonium formate (catalogue n. 70,221) of mass spectrometry grade were obtained from Merck (Darmstadt, Germany).

Polyvinylidene difluoride (PVDF; catalogue n. GEH10600021) and nitrocellulose membranes (catalogue n GEH10600001) were from GE Healthcare (Milan, Italy).

Fuji Super RX films (catalogue n. 47,410–19,284), FujiFilm Man-X Developer (catalogue n. 949–966) and FujiFilm Man-X Fixer (catalogue n. 949–974) were from Laboratorio Elettronico Di Precisione (Naples, Italy).

Experimental Design

Male Wistar rats (30 days old, P30) were purchased from Charles River; Calco, Como, Italy. Animals were caged individually in a temperature-controlled room (23 ± 1 °C) with a 12-h light/dark cycle (06.30–18.30), and divided into two groups, one fed a fructose rich diet (F group), the other one fed a control diet (C group) for 3 weeks. At the end of treatment, half of the rats from each group was euthanized, while the other half received a control diet (FR and CR groups) for further 3 weeks (P72). The composition of both control and fructose rich diet is reported in Table 1. The fructose-rich diet and the control diet were isocaloric, as differing only for qualitative content of carbohydrates (Table 1).

At the end of dietary treatment, the animals were then euthanized by decapitation, and frontal cortex was harvested and dissected as previously described [25]. In details, the brains were quickly harvested, moved on a metal plate placed in dry ice, and washed with cold PBS to remove surface blood. In order to dissect frontal cortex, brain was cut longitudinally, into right and left hemisphere, and the olfactory bulb was removed with a cut from the medial view of the hemisphere. Finally, frontal cortex was dissected from a slice about 2.5–4.5 mm anterior to bregma, taking into account published stereotaxic atlas resources [30, 31]. Pieces of each sample were immediately snap frozen in liquid nitrogen and stored at − 80 °C for metabolomic and protein analyses. Mitochondrial oxygen consumption was immediately assessed in little aliquots of tissue as reported below. Pieces of each

Table 1 Ingredients and nutritional composition of experimental diets

Ingredients, g/100 g	Control diet	Fructose diet
Standard Chow[a]	50.5	50.5
Sunflower Oil	1.5	1.5
Casein	9.2	9.2
Alphacel	9.8	9.8
Cornstarch	20.4	––
Fructose	––	20.4
Water	6.4	6.4
AIN-76 mineral mix	1.6	1.6
AIN-76 vitamin mix	0.4	0.4
Choline	0.1	0.1
Methionine	0.1	0.1
Energy content and composition		
Gross Energy Density (kJ/g)	17.2	17.2
ME content (kJ/g)[b]	11.1	11.1
Proteins (% ME)	29.0	29.0
Lipids (% ME)	10.6	10.6
Carbohydrates (% ME)	60.4	60.4
Of which:		
Fructose	––	30.0
Starch	52.8	22.8
Sugars	7.6	7.6

[a]4RF21, Mucedola, Italy

[b] Estimated by computation using values (kJ/g) for energy content as follows: proteins 16.736, lipids 37.656, and carbohydrates 16.736. ME = metabolizable energy

sample were fixed for immunofluorescence analysis. As post-mortem metabolism is known to provoke rapid and progressive changes in the levels of many compounds [32, 33], dissection of frontal cortex from each animal was performed into 1.5 min. The time of handling before freezing was standardized to minimize the effect of post-mortem metabolism.

Mitochondrial Analyses

Freshly isolated frontal cortex samples were homogenized (1:1000, w/v) in Mir05 medium containing 110 mM sucrose, 60 mM K-lactobionate, 20 mM Hepes, 20 mM taurine, 10 mM KH_2PO_4, 6 mM $MgCl_2$, 0.5 mM EGTA, and 0.1% w/v fatty acid-free BSA, pH 7.0.

Homogenates (2 mg) were transferred into calibrated Oxygraph-2 k (O2k, Oroboros Intruments, Innsbruck, Austria) 2-mL chambers. Oxygen polarography was performed at 37 ± 0.001 °C (electronic Peltier regulation), and oxygen concentration (μM) and oxygen flux (pmol O_2 s^{-1} mL^{-1}) were real-time recorded and corrected automatically for instrumental background by DatLab software (Oroboros Intruments, Innsbruck, Austria).

After addition of the homogenates, the O_2 flux was allowed to stabilize. A substrate, uncoupler, inhibitor titration (SUIT) protocol was applied to assess qualitative and quantitative mitochondrial changes [34]. After stabilization, leak respiration supported primarily by electron flow through complex I of the respiratory chain was evaluated by adding the substrates malate (0.5 mM), pyruvate (5 mM), and glutamate (10 mM). Electron transfer was coupled to phosphorylation by the addition of 2.5 mM ADP, assessing phosphorylating respiration with electron transfer supported by complex I. Succinate (10 mM) was added to the chamber to induce maximal phosphorylating respiration with parallel electron input from complexes I and II. Oligomycin (2.5 mM) was added to assess leak respiration when substrates and ADP were provided, but ATP synthase was inhibited. Maximum capacity of the electron transport chain was obtained by addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 0.5 mM). Rotenone (0.5 μM) was added to inhibit complex I; hence, the maximal capacity supported by complex II alone was determined. Residual oxygen consumption was established by addition of the inhibitor antimycin A (2.5 mM) and the resulting value was subtracted from the fluxes in each run, to correct for non-mitochondrial respiration. All samples were run in duplicates and the mean was used for analysis.

Procedures to test mitochondrial integrity were routinely carried out at the beginning of each measurement, by evaluating the stimulating effect of 10 mM exogenous cytochrome c on mitochondrial respiration in the presence of complex I- linked substrates and ADP.

Metabolic Parameter Assay

The amount of fructose, uric acid, and glucose in frontal cortex samples were measured by colorimetric enzymatic methods, using commercial kits according to the manufacturer's instruction (Fructose assay kit: catalogue n. FA-20, Sigma Aldrich, St. Louis, MO, USA; Uric acid kit: catalogue n. 4059, GS Diagnostics SRL, Guidonia Montecelio, Rome, Italy; Glucose assay kit: catalogue n. 4058, GS Diagnostic).

Protein Extraction

Aliquots of frontal cortex (about 50 mg) were homogenized in seven volumes (w/v) of cold RIPA buffer, as previously published [35]. Protein concentration was titrated by the colorimetric Bio-Rad assay, based on the Bradford method [36], using the Bio-Rad dye reagent (catalogue n. 5,000,006, Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. Then, protein extracts were used for titrating the markers reported below by ELISA or Western blotting.

Analysis of Tumor Necrosis Factor Alpha (TNF-Alpha)

TNF-alpha concentration was evaluated by sandwich ELISA in frontal cortex homogenates diluted 1:20 [37] using the TNF-alpha Duo-Set kit (catalogue n. DY510, R&D, DBA Italia). Data were reported as pg of TNF-alpha per mg of total proteins.

Western Blotting

Aliquots (30 µg) of cortex proteins were resolved by electrophoresis, under denaturing and reducing conditions [38], on 12.5% (to quantify glucose transporter-5, Glut-5; glucose transporter-4, Glut-4; glial fibrillary acidic protein, GFAP; synaptophysin; brain derived neurotrophic factor, BDNF; nerve growth factor, NGF; extracellular signal-regulated kinase, Erk1/2) or 10% (post-synaptic density protein 95, PSD-95; synaptotagmin I; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1α; nerve growth factor (NGF) receptor, p75NTR; tropomyosin receptor kinase B, TrkB; tropomyosin receptor kinase A, TrkA; thyroxine hydroxylase, TH) polyacrylamide gels. Proteins were then blotted onto PVDF or nitrocellulose membrane [39], and the following washing and blocking steps were performed according to [40].

The membranes were then incubated (overnight, at 4 °C) with primary antibody dilutions, followed by incubation (1 h, at 37 °C) with the appropriate peroxidase-conjugated secondary IgGs (see Supplementary Table 1).

For loading control, β-actin or vinculin was revealed after detection of each marker. To this aim, the membranes were stripped [41] and then treated with mouse anti-β-actin IgG or with mouse anti-vinculin IgG (overnight, at 4 °C) as described in Supplementary Table 1.

The Excellent Chemiluminescent detection Kit (Westar Antares, catalogue n. XLS142, Cyanagen s.r.l., Bologna, Italy) was used for detection. Chemidoc or digital images of X-ray films exposed to immunostained membranes were used for densitometric analysis and quantification was carried out by Un-Scan-It gel software (Silk Scientific, UT, USA).

Haptoglobin (Hpt) Evaluation

Hpt concentration in frontal cortex samples was measured by ELISA as previously reported [40]. Samples were diluted (1: 3,000, 1:10,000, 1:30,000) with coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6), and aliquots (50 µl) were then incubated (overnight, at 4 °C) in the wells of a microtiter plate (Nunc MaxiSorp, catalogue n. 44–2404-21, Thermo Fisher Scientific). Washing and blocking were carried out as previously reported [42], then the wells were incubated (1 h, at 37 °C) with 50 µl of rabbit anti-human Haptoglobin IgG (catalogue n. H8636, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:500 in T-TBS (130 mM NaCl, 20 mM Tris–HCl, 0.05% Tween, pH 7.4) containing 0.25% BSA, followed by 60 µl of goat anti-rabbit horseradish peroxidase-conjugated IgG (catalogue n. GtxRb-003-DHRPX, Immunoreagents, Raleigh, NC, USA; 1:5000 dilution in T-TBS containing 0.25% BSA; 1 h, at 37 °C). Peroxidase-catalyzed color development from o-phenylenediamine was measured at 492 nm.

Evaluation of Nitro-Tyrosine Levels, Acetylcholinesterase (AChE) and Monoamine Oxidase (MAO) Activities

Nitro-tyrosine (N-Tyr) titration was carried out by ELISA in frontal cortex homogenates as previously described [43]. Samples were diluted (1:1,500, 1:3,000, 1:6,000) with coating buffer, and aliquots (50 µl) were then incubated in the wells of a microtiter plate (overnight, at 4 °C). After washing and blocking, the wells were incubated (1 h, 37 °C) with 50 µl of rabbit anti-N-Tyr IgG (catalogue n. CVL-PAB0188, Covalab, distributed by VinciBiochem, Vinci, Italy; 1: 1000 dilution in T-TBS containing 0.25% w/v BSA) followed by 60 µl of goat anti-rabbit horseradish peroxidase-conjugated IgG (1:9,000 dilution; 1 h, at 37 °C). Peroxidase-catalyzed color development from *o*-phenylenediamine was measured at 492 nm. Data were reported as OD per milligram of total proteins.

The acetylcholinesterase (AChE) activity was measured in frontal cortex samples as previously described [25]. Enzyme activity was expressed as nmol/min mg protein.

The monoamine oxidase (MAO) activity was measured spectrophotometrically following the conversion of benzylamine to benzaldehyde, as previously described [44].

Immunofluorescence Analysis

Paraffin embedded sections of frontal cortex from all the groups were stained with the phospho-cAMP response element-binding protein (p-CREB) specific monoclonal antibody (Ser 133) (87G-3) (catalogue n. 9198, Cell Signaling Technology; 1:1,000 in dilution in PBS containing 2% w/v BSA; overnight, at 4 °C), and DAPI (catalogue n. D9542, Sigma Aldrich, Saint Louis, MO, USA). For the analysis, images were acquired with × 40 magnification and 3 random fields/section per rat were analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Images were captured and visualized using a Nikon Eclipse E1000 microscope.

Liquid chromatography high resolution tandem mass spectrometry (LC–MS/MS)

Polar hydrophilic compounds were analyzed by liquid chromatography high resolution tandem mass

spectrometry (LC–MS/MS) as previously reported [45], with minor modifications. Frontal cortex samples (25 ± 10 mg) were dissolved in 0.390 mL of 0.1% formic acid along with 10 µL of 10 µg/mL lysine *d-4*; suspensions were accurately homogenized by using a stainless-steel disperser (IKA T10, Staufen, Germany, 3 passes, 30 s) in an ice bath. Supernatants (0.1 mL) were further purified by directly using 0.3 mL of 0.1% formic acid in acetonitrile in a protein precipitation and phospholipids removal cartridge (Phree, 1 mL, Phenomenex, Torrance, CA); eluates were collected and dried by using a centrifugal evaporator (SpeedVac, Thermo Fisher Scientific, Bremen, Germany). Dried samples were dissolved in 0.1 mL of a mixture acetonitrile: water: formic acid (50:49.9:0.1, v/v/v) and 5 µL injected into the LC–MS/MS system consisting in a linear ion trap with Orbitrap detector (LTQ Orbitrap XL) interfaced to an Ultimate 3000 RS (Thermo Fisher Scientific, Bremen, Germany). Analytes were separated through hydrophilic interaction chromatography and analyzed in data dependent scan positive ions mode for identification and quantitation.

Chromatographic separation was achieved through a silica sulfobetaine zwitterionic modified HILIC column (100 × 2.1 mm, 1.7 µm, Syncronis HILIC, Thermo Fisher, Bremen, Germany) at 35 °C. Mobile phases consisted in 0.1% formic acid in acetonitrile:water 95:5 (v/v, solvent A) and 0.1% formic acid in water (solvent B) both with 5 mM ammonium formate. Analytes (thermostated at 4 °C) were separated through the following gradient of solvent B (minutes/%B): (0/3), (3.5/3), (15.5/75), (17.5/75) at a flow rate of 0.25 mL/min. Electrospray interface (ESI) parameters were the following: spray voltage 5.0 kV, capillary voltage 21.0 V, capillary temperature 300 °C, sheath gas flow and auxiliary gas flow were 25 and 4 arbitrary units, respectively. Profile data type were acquired in full scan FTMS mode (Fourier transformed) in the mass range 75–750 *m/z*. For data-dependent scanning mode, MS/MS normalized collision energy was set to 20, activation Q 0.25, activation time 25 ms, with a 1 *m/z* isolation window, while a reject mass list was generated by injecting blank samples consisting in a mixture of acetonitrile:water:formic acid (50:49.9:0.1, v/v/v).

For compound identification, differential analysis, principal component analysis (PCA), hierarchical clustering, and identification of metabolic pathways, raw data were loaded in Compound Discoverer (v. 3.2, Thermo Fisher Scientific). The workflow included the identification of both expected and unknown metabolites; briefly, each node performed retention time alignment, expected compound detection, biotransformation, dealkylation, and dearylation products formation. Resolution and isotope pattern matching with unknown compounds detection were used across all samples with a mass accuracy below 5 ppm. FISh (fragment ion searching) scoring was applied to all expected compounds with automatic fragment annotations based on targeted and untargeted compound chemical behavior outlined in Human Metabolome Database (https://hmdb.ca/), ChemSpider (http://www.chemspider.com), mzCloud (https://www.mzcloud.org/) and KEGG pathway database (https://www.genome.jp/kegg/compound/).

According to the background in blank samples, the procedure predicted elemental compositions for all unknown compounds, while quality control samples (QC, consisting in pooled samples spiked with amino acid standards) corrected signal intensities each 10 runs. Hierarchical clustering was obtained through filtering procedures based on technical replicates coefficient of variation (CV%), retention time, and mass accuracy, excluding analytes eluting close to the solvent front, with poor response for coefficient of separation *k'*, peak shape, and spectrum purity in both full scan and data-dependent mode. The workflow included differential analysis (*p*-values, adjusted *p*-values, ratios, fold change; Supplementary Tables 2 and 3), Euclidean distance, and complete linkage method without data normalization to enhance differences among frontal cortex of groups fed different diets. Analytes based on hierarchical clustering were scaled before clustering through a z-score transformation.

For targeted analyte quantitation, a calibration curve of the compounds listed in Supplementary Table 4 was built in the range 100–5000 ng/mL by using lysine-*d4* as internal standard. Linearity and the responses of intraday and interday assays were monitored by using Xcalibur 2.1 with a mass accuracy fixed at 5 ppm (Thermo Fisher Scientific, Bremen).

Statistical Analysis

Data were expressed as mean values ± SD. The program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) was used to verify normal distribution of data by Shapiro–Wilk normality test and to compare groups with one-way ANOVA followed by Bonferroni post-test. $P < 0.05$ was considered significant in the reported analyses.

Results

Metabolic Analyses

At the end of dietary treatment, no significant variation was evident in final body weight (C rats = 295 ± 4 g; F rats = 292 ± 5 g; CR rats = 350 ± 6 g; FR rats = 367 ± 3 g). Daily energy intake showed no significant variation in F rats compared to C rats (C rats = 358 ± 5 kJ/day; F rats = 360 ± 5 kJ/day). Similarly, comparable energy intake was found in CR and FR rats (CR rats = 340 ± 4 kJ/day; FR rats = 344 ± 5 kJ/day). In addition, fasting levels of glucose were unchanged both in F rats compared to C rats (F rats = 123 ± 5 mg/dL; C rats = 130 ± 5 mg/dL), and in FR rats compared to CR rats (FR rats = 113 ± 6 mg/dL; CR

rats = 121 ± 6 mg/dL). These data evidence that the impact of fructose on brain is not due to an increase of body weight or change in daily energy intake.

Inflammation, Oxidative Stress, and Mitochondrial Activity

To investigate whether the dietary treatment induces fructose transporter and metabolism, the protein expression of Glut-5, as well as the level of fructose and uric acid, one of the main products of fructose metabolism, was analyzed in frontal cortex after 3 weeks of a fructose-rich diet. The amount of Glut-5 was significantly higher [F (3, 28) = 7.625; $P < 0.01$] in frontal cortex of F rats compared to C rats, while this increase disappeared in FR rats (Fig. 1a). Unlike Glut-5, no significant difference was found in the level of Glut-4 between C and F rats or CR and FR (Supplementary Figure 1). According to the Glut-5 increase, a significant rise in the levels of fructose [F (3, 28) = 5.191; $P < 0.01$] and uric acid [F (3, 28) = 6.258; $P < 0.01$] was found in F rats compared to C rats, while no significant differences were found in FR rats compared to CR ones (Fig. 1b, c). The cortex level of glucose did not differ between C and F, or CR and FR rats (Supplementary Figure 1).

We then investigated the inflammatory status by measuring the protein expression of GFAP, a marker of astrogliosis, the pro-inflammatory cytokine TNF-alpha, and Hpt, a marker of inflammation very sensitive to nutritional changes [37, 40, 46]. As shown in Fig. 1d–f, an increase of inflammatory markers in frontal cortex was associated with the fructose-rich diet. As a matter of the fact, significantly higher levels of GFAP [F (3, 28) = 4.979; $P < 0.01$; Fig. 1d], TNF-alpha [F (3, 28) = 11.57; $P < 0.01$; Fig. 1e], and Hpt [F (3, 28) = 5.656; $P < 0.01$; Fig. 1f] were found in frontal cortex of F rats compared to C rats. Importantly, these conditions were rescued after switching to a control diet as no difference in the levels of these markers was detected between CR and FR rats.

In line with previous results obtained in the hippocampus [47], fructose feeding was also associated with an increase of N-Tyr, the footprint of protein oxidative damage induced by peroxynitrite [48] in fructose-fed rats [F (3, 28) = 7.283; $P < 0.001$; Fig. 1g]. The observed condition of redox imbalance was corroborated by changes in mitochondrial activity induced by fructose-rich diet. In detail, F rats showed a significant decrease in leak respiration with complex I and II-linked substrates [F(3, 28) = 10.36; $P < 0.0001$], in ADP-supported respiration with complex I [F (3,28) = 13.58; $P < 0.0001$] or complex I- and II-linked substrates [F (3, 28) = 11,61; $P < 0.0001$] and in FCCP-stimulated respiration with complex II [F (3, 28) = 52.51; $P < 0.0001$] or complex I and II-linked substrates [F (3, 28) = 3.70; $P = 0.023$; Fig. 1l]. The above mentioned mitochondrial dysfunction was reversed when rats were switched to a control diet. The analysis of PGC-1α was performed as a marker of mitogenesis, and no difference was observed (Fig. 1m).

We also detected higher levels of *Nε*-carboxymethyllysine (CML) [F (3, 28) = 10.62; $P < 0.01$; Fig. 1h] and *Nε*-carboxyethyllysine (CEL) [F (3, 28) = 7,756; $P < 0.05$; Fig. 1i], two advanced glycation end-products [49], both in free form, in frontal cortex of F rats compared to C rats. Notably, after switching to the control diet, the differences in N-Tyr between CR and FR rats disappeared, while CML and CEL levels persisted higher in FR compared to CR rats ($P < 0.01$).

Neurotrophins and Synaptic Proteins

The level of BDNF, a key cerebral factor involved in a wide range of neurophysiological processes such as neuronal survival, synaptic transmission and plasticity [50], was measured in the frontal cortex of all groups of rats. As shown in Fig. 2a, a significant fructose diet-dependent decrease of the mature form of BDNF was observed [F (3, 28) = 11.84; $P < 0.01$]. Notably, alterations of BDNF amount persisted after switching to a control diet, as displayed by FR rats compared to CR rats ($P < 0.001$). No difference was found in the levels of BDNF precursor (pro-BDNF), suggesting that changes in this neurotrophin are likely at post-translational level. The protein amount of TrkB, the high-affinity receptor of BDNF, was also analyzed, as its activation enhances synaptic plasticity, neuroprotection, and neurite outgrowth [50]. As shown in Fig. 2b, TrkB level was significantly lower in the frontal cortex of F rats compared to C ones [F (3, 28) = 3.994; $P < 0.05$], and its level in FR rats returned to values comparable to CR rats. We further investigated the extent of phosphorylation of CREB, the major downstream effector of BDNF [51, 52], as marker of BDNF signalling. In line with the decrease of BDNF, a decrease in the activating phosphorylation of CREB (p-CREB) was found in both F rats compared to C, and FR compared to CR [F (3, 28) = 12.42; $P < 0.01$; Fig. 2c], as assessed by immunofluorescence.

According to previous works in human, rat, and mouse brain, reporting that mature NGF is absent while pro-NGF is the predominant species [53, 54], mature NGF was not detectable in any frontal cortex samples analyzed. Moreover, no significant difference between the experimental groups was found in the levels of its precursor pro-NGF (Fig. 3a). Interestingly, the protein level of TrkA, the receptor of NGF, was increased in F rats respect to C [F (3, 28) = 3.769; $P < 0.05$], while no difference was detected in FR compared to CR, after the sugar removal from the diet (Fig. 3b).

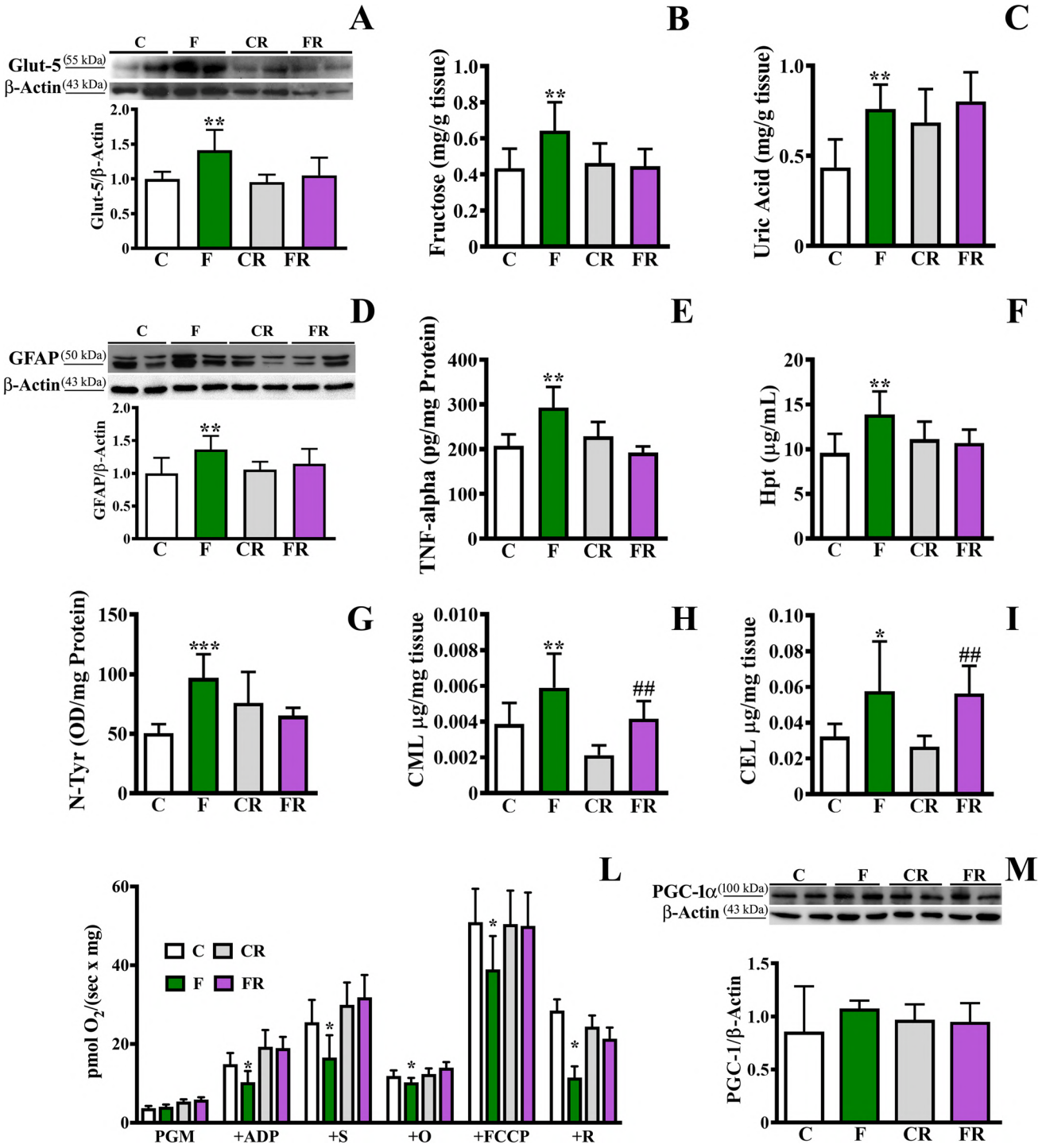

Fig. 1 Evaluation of Glut-5, fructose, uric acid, inflammatory and oxidative stress markers, mitochondrial function in frontal cortex. (**a**) Glut-5 level (representative western blot and densitometric analysis); (**b**) fructose amount; (**c**) uric acid amount; (**d**) GFAP amount (representative western blot and densitometric analysis); (**e**) TNF-alpha concentration (titrated by sandwich ELISA); (**f**) Hpt concentration (titrated by ELISA, (**g**); N-Tyr levels (titrated by ELISA); (**h**) CML amount and (**i**) CEL amount (as determined by LC–MS/MS); (**l**) non-normalized respiration after addition of malate + pyruvate + glutamate (PMG), ADP, succinate (S), oligomycin (O), FCCP, and rotenone (R); (**m**) PGC-1α amount (representative western blot and densitometric analysis), in frontal cortex of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Data are the means ± SD of 8 rats/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus C rats. $\#\#P < 0.01$ versus CR rats. Source of variation: one-way ANOVA followed by Bonferroni post-test. FCCP carbonyl cyanide *p*- trifluoromethoxyphenylhydrazone

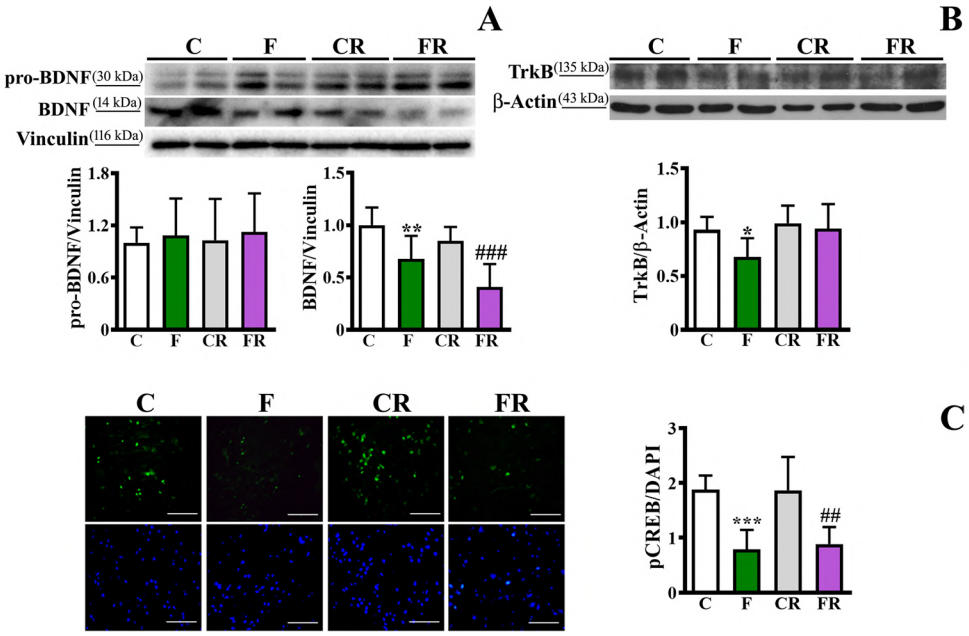

Fig. 2 Evaluation of BDNF, TrkB and pCREB amount in frontal cortex. (**a**) pro-BDNF and BDNF levels (representative western blot and densitometric analysis), (**b**) TrkB level (representative western blot and densitometric analysis), (**c**) immunofluorescence of p-CREB (magnification 40×, scale bar = 50 µm) in frontal cortex of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Data are the means ± SD of 8 rats/group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus C rats. $^{\#\#}P < 0.01$ versus CR rats. Source of variation: one-way Anova followed by Bonferroni post-test

Furthermore, no changes were observed in the amount of the low-affinity NGF receptor p75NTR (Fig. 3c). In addition, the phosphorylation degree of both Erk1 [F (3, 28) = 7.93; $P < 0.01$], and Erk2 [F (3, 28) = 14.16; $P < 0.001$], significantly increased in F compared to C rats (Fig. 3d), with persistent increased levels in FR compared to CR rats ($P < 0.01$ and $P < 0.001$, respectively).

We further investigated the impact of fructose on the amounts of two pre-synaptic proteins, namely synaptophysin and synaptotagmin I, and the post-synaptic protein PSD-95, which play a key role in synaptic plasticity [55]. The fructose-rich diet led to decreased levels of all the three proteins [synaptophysin, F (3, 28) = 6.601; $P < 0.001$; synaptotagmin I, F (3, 28) = 6.978; $P < 0.01$; PSD-95, F (3, 28) = 5.452; $P < 0.01$], while the switch to control diet rescued their amount (Fig. 4a–c).

The effect of the short-term fructose diet on the activity of AChE, a pivotal enzyme involved in the regulation of cholinergic pathway [56], was also evaluated. As shown in Fig. 4d, fructose feeding resulted in a significant increase of AChE activity of F compared to C rats [F (3, 28) = 4.36; $P < 0.05$]. This increase was also found in FR rats compared to CR ($P < 0.05$). Notably, the activity of MAO, a central player in the modulation of monoamine neurotransmitters level [57], increased in F rats compared to C rats [F (3, 28) = 29.12; $P < 0.0001$; Fig. 4e), with no significant change between FR and CR rats.

Metabolites and Neurotransmitters

Polar hydrophilic metabolites were isolated from frontal cortex homogenates, purified by using protein and phospholipids cartridges, and finally measured by using HILIC coupled to high-resolution tandem mass spectrometry according to the nature and duration of the intervention study. To point out the role of fructose intake on small molecules fingerprinting, the C rat group was compared with the F one, while CR was matched to FR. Results are reported in the heat-maps (Fig. 5). Figure 5a highlighted a clear discrimination between fructose-diet (orange line) and control diet (blue line) through Euclidean distance between over- and down-represented analyte intensities in the centred and scaled area range − 2.8 and 3.9, as related to the *m/z* current ion associated with 89 molecules. Supplementary Table 2 shows over-represented compounds reported in Fig. 5c as well as the corresponding analytical performance and fold change values, along with their respective MSI (metabolomic standard initiative) levels [58]. Sample clustering was outlined in an explorative PCA combined with loading plots and differential analysis, which highlighted (in light blue) the compounds (Supplementary Table 2) having a log2 fold change higher than 0.5 and a negative log10 of the *p*-value higher than 0.05 (Supplementary Figure 2a).

In a similar way, polar hydrophilic metabolites differentiated rescue fructose-diet (light blue arrow, FR) and rescue control diet (blue arrow, CR) (Fig. 5b) when considering up to 272 over- and down-represented compounds. Supplementary Table 3 shows over-represented compounds in FR rats reported in Fig. 5d with a log2 fold change higher than 0.5 and with a negative log10 of the *p*-value higher than 0.05, as a result of the differential analysis (Supplementary Figure 2b). PCA illustrates how loadings plot contributed to the spatial arrangement of FR and CR samples, confirming

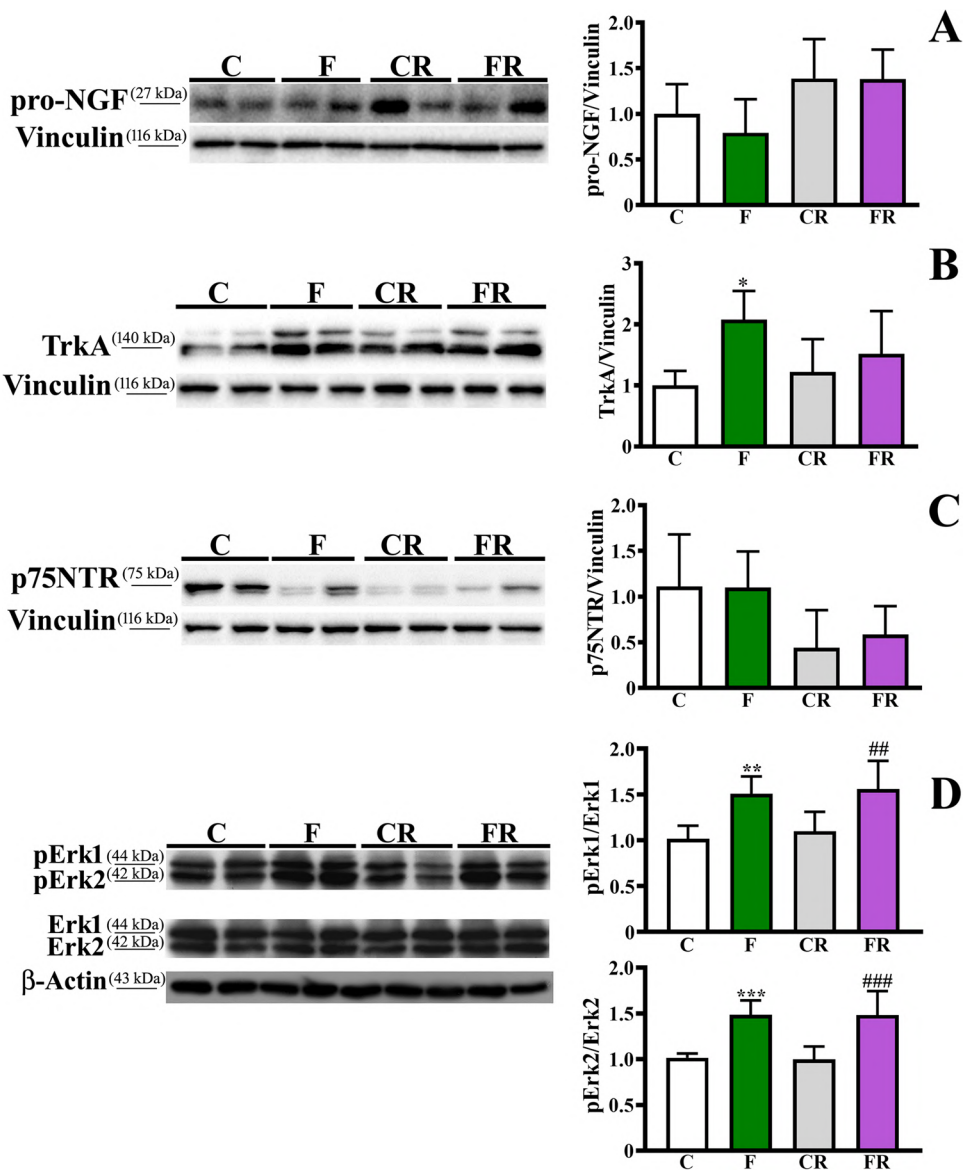

Fig. 3 Evaluation of pro-NGF, TrkA, p75NTR, and Erk1/2 in frontal cortex. Representative western blot and densitometric analysis of (**a**) NGF level, (**b**) TrkA level, (**c**) p75NTR level, (**d**) pErk1/2/Erk1/2 ratio, in frontal cortex of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Data are the means ± SD of 8 rats/group. $*P<0.01$, $**P<0.01$, $***P<0.001$ versus C rats. $\#\#P<0{,}01$, $\#\#\#P<0.001$ versus CR rats. Source of variation: one-way Anova followed by Bonferroni post-test

results obtained through heat-map and hierarchical clustering (Supplementary Figure 2b).

Then, each of the over-represented compounds differentiated by the kind of diet (Supplementary Tables 2 and 3) was included in Metabolika node within Compound Discoverer with the aim to identify the most influenced metabolic pathways. Results obtained after loading the increased abundance of metabolites in Fig. 5c pinpointed metabolic pathways involving aromatic amino acids, namely tyrosine, phenylalanine, and tryptophan, along with sulphur compounds (cysteine and reduced glutathione) and other derivatives.

On the other hand, loading of data from the second heat-map deriving from differential analysis on rescue diet (Fig. 5d) outlined metabolic pathways associated with some polar basic amino acids, such as lysine and CEL, secondary amides and other acid compounds, like aspartic acid derivatives and glutamic acid (Fig. 5d). In this view, we hypothesized that the metabolism of aromatic amino acids, lysine, and glutamic acid can be the focus of high fructose diet. This hypothesis is in accordance with a previous observation [59], which reported that amino acids linked to tricarboxylic acid cycle, such glutamate and aspartate, undergo specific enrichment when young rats are fed with a high-fructose diet. Furthermore, a coherent alteration of the lysine catabolism was recently observed in diurnal metabolic pathways impacted by high-fat diet, suggesting that fructose intake can similarly exacerbate the alteration of amino acid metabolism [60].

Based on the above-reported untargeted analysis (Fig. 5), we then focused on the targeted quantification of six metabolites, based on full scan high resolution acquisition and the use of authentic reference standards,

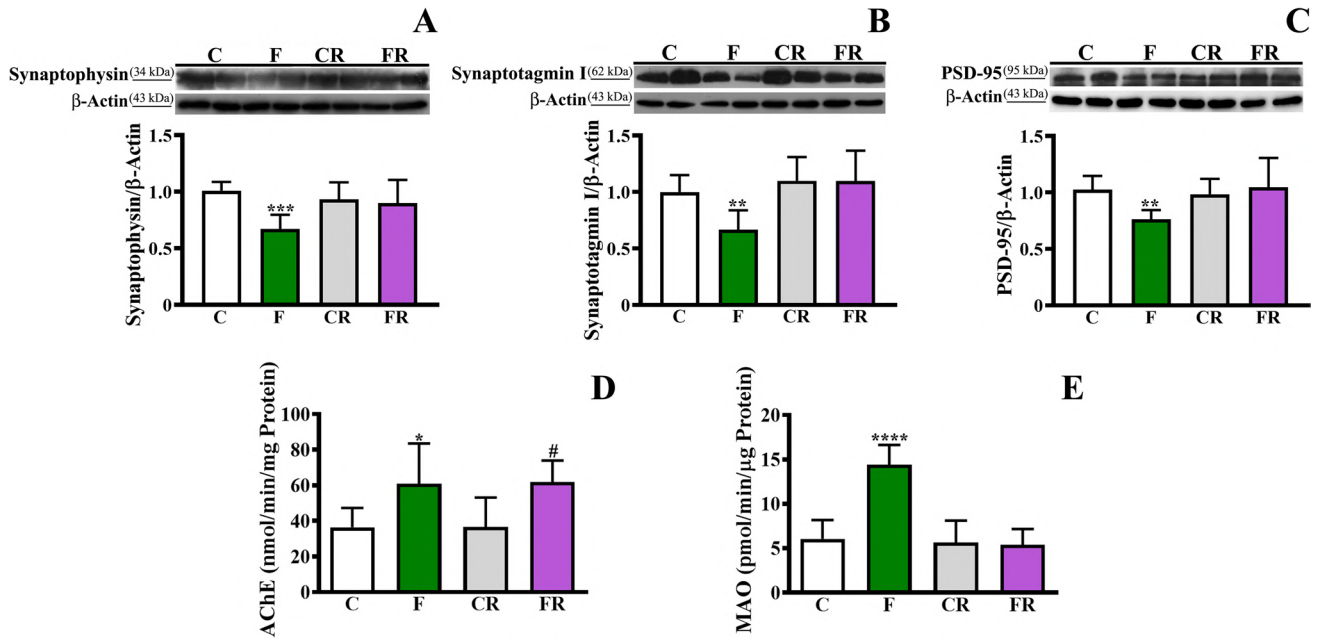

Fig. 4 Evaluation of synaptic proteins, acetylcholinesterase and monoamino oxidase activity in frontal cortex. (**a**) Synapthophysin level (representative western blot and densitometric analysis), (**b**) synaptotagmin I level (representative western blot and densitometric analysis), (**c**) PSD-95 level (representative western blot and densitometric analysis), (**d**) AChE activity, and (**e**) MAO activity in frontal cortex of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Data are the means ± SD of 8 rats/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$ versus C rats. $\#P < 0.05$ versus CR rats. Source of variation: one-way Anova followed by Bonferroni post-test

to highlight quantitative differences between C and F rats, and between CR and FR. Indeed, a major aim of the study was to evaluate the quantitative impact of the fructose diet on the profile of the major neurotransmitters (Fig. 6). We observed that the concentration of acetylcholine (ACh), one of the main neuromodulators of the central nervous system regulating individual attention, learning, and memory [61], was significantly higher in F rats compared to C [F (3, 20) = 4.202; $P < 0.01$], and this increase was rescued in FR (Fig. 6a). Conversely, the levels of dopamine, the neurotransmitter regulating the food eating reward circuit, motor activity, and emotion [62, 63], were reduced in F rats compared to C ones [F (3, 20) = 11.62; $P < 0.001$; Fig. 6b], and no difference between CR and FR groups was observed. Similarly, levels of both dopamine precursors tyrosine and tyramine decreased in F compared to C rats [tyrosine, F (3, 20) = 6.788, $P < 0.01$; tyramine, F (3, 20) = 6.509, $P < 0.05$; Fig. 6c–d], and returned to control values after the switch to the standard diet. On the other hand, no changes in the amounts of TH, the enzyme catalysing the rate-limiting step of catecholamine biosynthesis [64], were observed between F and C groups (Fig. 6e). Interestingly, the levels of the most common neurotransmitter in the CNS, namely glutamate [65], were lower in F group with respect to C one [F (3, 20) = 7.047; $P < 0.01$; Fig. 6f]. A different trend was observed after the switch to control diet, as FR showed higher glutamate levels compared to CR (Fig. 6f; $P < 0.01$). These results demonstrated that fructose intake is associated with a dysregulation of glutamate metabolism, which is further observed, but with an opposite quantitative trend, after switching to control diet. Finally, GABA levels did not differ between C and F groups, while increased amounts were observed in FR compared to CR [F (3, 20) = 3.477; $P < 0.01$; Fig. 6g].

Discussion

Different lines of evidence have recently highlighted the fructose impact on brain metabolic alterations [13, 23], but it remains unclear whether the observed sugar-induced adverse effects are limited exclusively to the period of increased intake or are persistent even when it is eliminated from the dietary regimen. We recently reported that the fructose-rich diet impacts different metabolic parameters by inducing systemic inflammation, hepatic insulin resistance, increase of plasma triglycerides [66], and "leaky gut" [67], which persisted after switching to control diet. Sugar-driven systemic inflammation and metabolic dysfunction could impact on brain as well. Here we studied, by using the same experimental design, the metabolic effects of the sugar in frontal cortex of adolescent animal

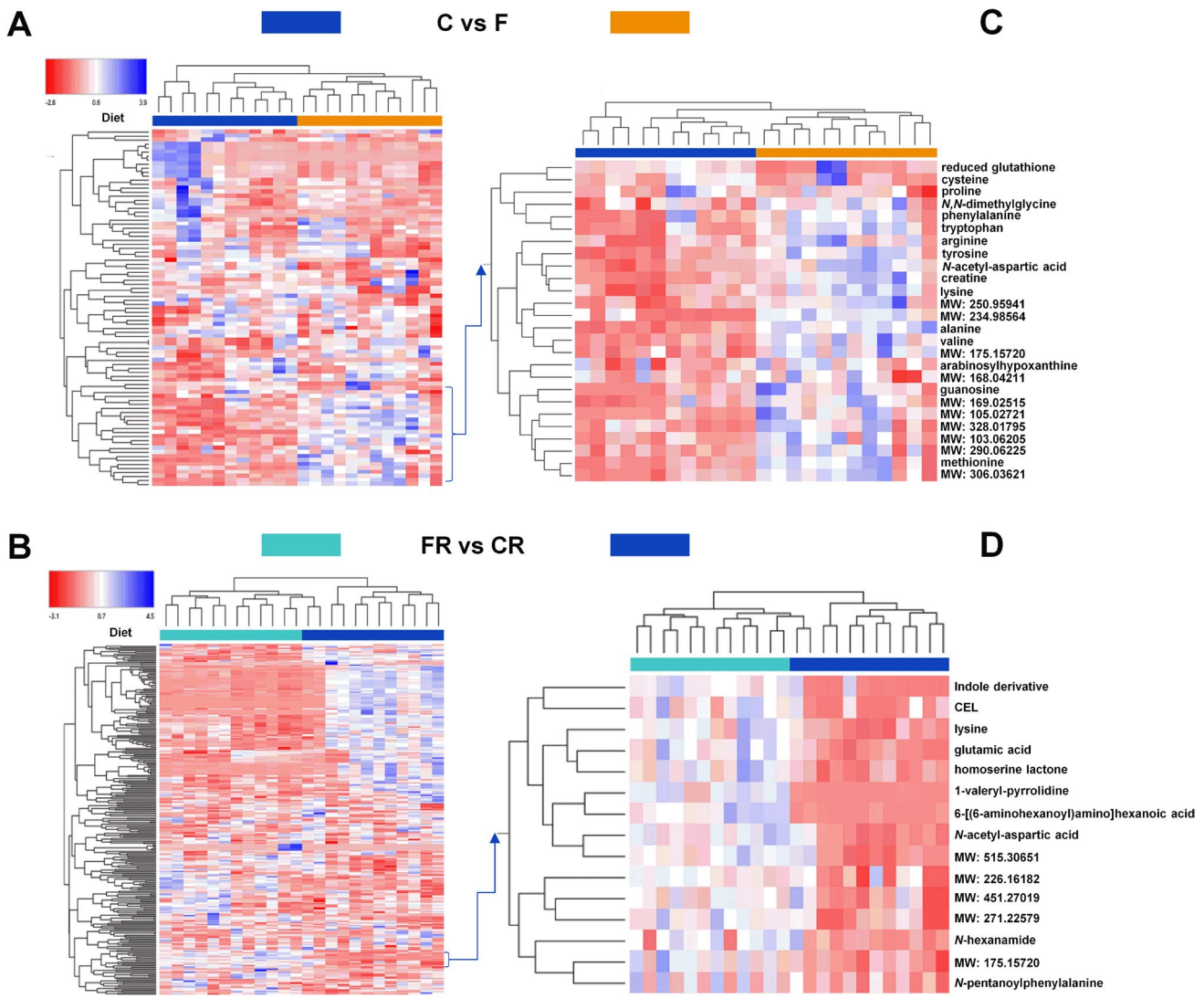

Fig. 5 Metabolomic analysis of frontal cortex. (**a**) Heat-map hierarchical cluster analysis of metabolites detected in frontal cortex of fructose-fed adolescent (F, orange) and control adolescent (C, blue) rats. (**b**) Heat-map hierarchical cluster analysis of metabolites detected in frontal cortex of young-adult fructose-rescued (FR, light blue) and young-adult control rescued (CR, blue) rats. Dendrograms on the left report the molecular grouping and distance between molecular classes, while dendrograms on the top report the sample grouping. Corresponding iterative zoomed regions on the right (panels **c** and **d**) report identified molecules according to the number of matched over-represented compounds as result of differential analysis processing node within Compound Discoverer software (Supplementary Figure 2, and Supplementary Tables 2 and 3). Analytes were identified with a metabolite identification level of 2 as in the case that compound name was used and 4 in the case where only the calculated molecular weight (MW) was reported, as defined by Metabolomics Standards Initiative (MSI) [58]. Normalized area ranges and Eculidean distance were used in all cases; scaled expression values of each range are plotted in red to blue through white color scale

model, since, to our knowledge, poor information is available on this issue.

The fructose diet was associated with increased levels of the fructose transporter Glut-5 in rat frontal cortex, and a concomitant increase of fructose and uric acid, which were suggestive of an enhanced fructose metabolism therein. The change of Glut-5 and metabolite levels were paralleled by an inflammatory status. A similar pattern was previously found in the hippocampus [47], thus showing that dietary fructose reaches the brain and is utilized in several cerebral areas. Augmented levels of uric acid were also reported to elicit oxidative stress [68–70] and consistently we found a higher extent of oxidative damage to proteins. Inflammation and oxidative stress are often related to a mitochondrial dysfunction [71, 72], and indeed we evidenced a corresponding general impairment of the mitochondrial oxidative capacity not linked to a lower organelle mass, since PGC-1α level was not altered. All above-mentioned fructose-dependent effects

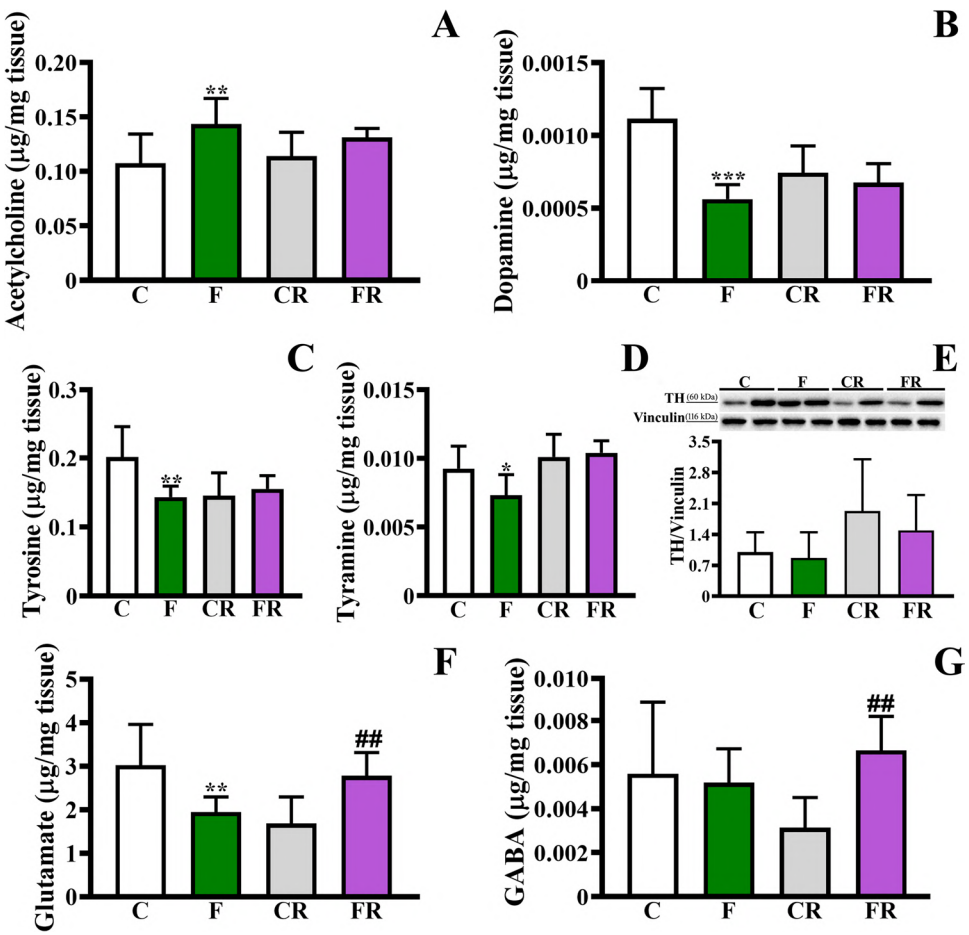

Fig. 6 Evaluation of neurotransmitters and tyroxine hydrolase in frontal cortex. (**a**) Acetylcholine amount, (**b**) dopamine amount, (**c**) tyrosine amount, (**d**) tyramine amount, (**e**) tyroxine hydroxylase level (representative western blot and densitometric analysis), (**f**) glutamate amount, (**g**) GABA amount in frontal cortex of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Data are the means ± SD of 6 rats/group. $*P < 0.01$, $**P < 0.01$, $***P < 0.001$ versus C rats. $\#\#P < 0{,}01$ versus CR. Source of variation: one-way Anova followed by Bonferroni post-test

on mitochondrial activity were reverted when this sugar was removed from the dietary regimen.

More importantly, higher advanced glycation end-products (CML and CEL) were found in fructose-fed rats, in line with previous works showing that fructose can lead to the formation of highly reactive intermediate products as α-dicarbonyls [73], which in turn can favor the formation of advanced glycation end-products, as CML and CEL [74], thus acting as potent glycating agent [75] and negatively impacting the brain function [13, 76, 77]. Interestingly, the observed augmented levels of CML and CEL in fructose-fed rats were not restored to control values in FR group, suggesting the persistence of the sugar-associated effects.

Given the key role of neurotrophins in brain activity [50], we focused on the analysis of BDNF and NGF and we observed that the level of BDNF and its receptor TrkB was lower in fructose-fed rats. This result is in line with previous findings obtained in adult rats experiencing a long- or short-term fructose intake [25, 27], highlighting this neurotrophin as a crucial target of fructose diet independently from the age. The early reduction of BDNF in adolescent rats and its persistent decrease later was corroborated by the observation of lower activation of its downstream effector, namely CREB, both in F compared to C rats and in FR compared to CR counterparts. Since CREB is implicated in the transcription of genes essential for synaptic plasticity [78], the persistent reduction in its phosphorylated form is suggestive of a possible long-term brain impairment. The sugar-induced impairment of BDNF signaling might be compensated, at least in part, by the increase of TrkA and activation of its downstream transducers Erk1/2. This process is regulated by pro-NGF and critically depends on the balance of TrkA and p75NTR [79, 80]. This pathway is indeed committed to prevent apoptotic signals, with a positive effect on neuronal survival and neurite outgrowth [54].

The persistent impairment of BDNF-CREB signaling prompted us to investigate the issue of synaptic functioning. A decrease of pre- (synaptophysin, and synaptotagmin I) and post-(PSD-95) synaptic proteins following fructose dietary treatment was found, and this change was rescued after the switch to the standard diet, suggesting that the homeostasis of these proteins is restored more rapidly than that of BDNF. To gain further insight into synaptic physiology, we also measured the activity of AChE and MAO, as well as the levels of important neurotransmitters involved in synaptic transmission. In particular, increased activity of

AChE and MAO was detected in fructose-fed rats. While increased MAO rescued after switching to standard diet, AChE increase persisted after fructose removal from the diet. In this context, it is worth mentioning that the enhanced AChE activity in the hippocampus and prefrontal cortex was previously proposed as an early event linked to hypercholesterolemia- or high-fat diet-induced alterations in cognitive function [81–83]. Notably, despite the enhanced AChE activity, we detected increased levels of ACh in fructose-fed rats. In this regard, previous studies showed that an abnormal intake of fructose provokes an immediate drop in the ATP/AMP ratio, a decreased acetyl-CoA carboxylase activity, and a consequent lowering of malonyl-CoA levels [84]. Accordingly, it can be hypothesized that the increased ACh levels after fructose intake might derive from the increased availability of acetyl CoA, consequent to the decreased activity of acetyl-CoA carboxylase. In this sense, the higher AChE activity may represent an adaptive response to prevent prolonged ACh signaling. The increase of ACh levels in F rats suggests that a short-term fructose enriched diet may disrupt cholinergic signaling and predispose adolescents to altered states, such as anxiety disorders and depression, also corroborated by BDNF and dopamine reduction. The observed decrease of dopamine, which regulates different brain functions such as reinforcement processing, motivation, and attention [85], might be ascribed to both the corresponding enhanced MAO activity and the reduced amounts of tyrosine and tyramine. In agreement with our findings, previous studies have reported that HFCS can impair dopamine function in the absence of weight gain or increased fat consumption [10]. As a reduced dopamine activity has been implicated in reduced energy expenditure [86, 87], changes in dopamine metabolism were proposed to precede and possibly contribute to obesity in the long-term period [10].

A general alteration of some metabolic pathways related to specific aromatic or sulfur-containing amino acids, lysine and glutamic acid was also deduced from F vs C, and FR vs CR comparisons. Glutamate is the principal excitatory neurotransmitter involved in learning, memory and cognition, and any unbalance of its turnover may have severe consequences [65]. Decreased glutamate levels in fructose-fed rats and then an increase of this neurotransmitter in FR rats compared to CR counterpart might be suggestive of a glutamatergic persistent dysregulation after fructose intake, which

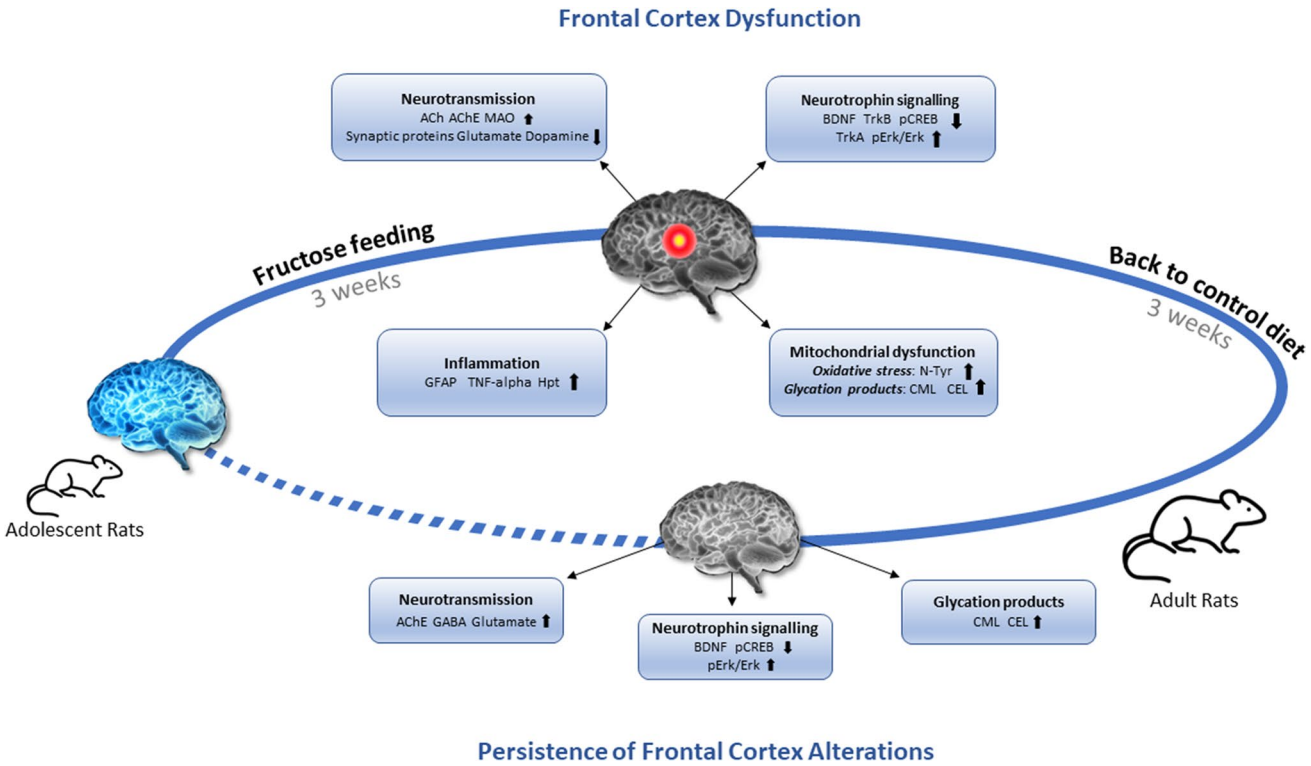

Fig. 7 Fructose impact on brain health of adolescent rats and persistence of its effect after switching to a control diet. Up arrows indicate parameters increased and down arrows indicate parameters reduced. ACh, acetylcholine; AChE, acetylcholinesterase; MAO, monoamine oxidase; BDNF, brain derived neurotrophic factor; TrkB, tropomyosin receptor kinase B; TrkA, tropomyosin receptor kinase A; pErk1/2, pospho-extracellular signal-regulated kinase 1/2; Erk1/2, extracellular signal-regulated kinase 1/2; pCREB, pospho-cAMP-response element binding protein; GFAP, glial fibrillary acidic protein; Hpt, haptoglobin; CML, *Nε*-carboxymethyllysine; CEL, *Nε*-carboxyethyllysine

might prelude to long term dysfunction. Indeed, glutamatergic dysregulation has already been reported being an important contributor to different neurological pathologies [65].

ACh, dopamine, and glutamate changes observed in fructose-fed rats were not paralleled by GABA alterations. Since higher levels of glutamate were observed in FR rats, we cannot exclude that the concomitant increase of GABA amount may represent a compensatory mechanism to prevent possible dysfunction related to concomitant quantitative changes of its precursor.

In conclusion, this study demonstrates that the fructose feeding causes a perturbation of various biochemical machineries involved in brain metabolism and function, such as neurotrophins signaling and a consequent possible modification of excitatory/inhibitory neurotransmitter balance, which is essential for the proper functioning of the central nervous system (Fig. 7). Undoubtedly, these data also point out that adolescence represents a developmental window of vulnerability, in which extreme attention should be devoted to limit an excessive consumption of industrial and processed sweet food, since it can impact brain physiology not only immediately but also in the long term. Since previous reports showed different susceptibilities of males and females to fructose supplementation [88, 89], future experiments will be critical to clarify whether the sugar-adverse effects and/or their persistence in brain can be different depending on the sex.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-022-03115-8.

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Author Contribution Luisa Cigliano, Maria Stefania Spagnuolo, Arianna Mazzoli, and Susanna Iossa contributed to the study conception and design. Material preparation was performed by Maria Stefania Spagnuolo, Arianna Mazzoli, Martina Nazzaro, and Luisa Cigliano. Data collection and analysis were performed by Maria Stefania Spagnuolo, Arianna Mazzoli, Martina Nazzaro, Cristina Gatto, Antonio Dario Troise, Claudia Tonini, Mayra Colardo, Marco Segatto, Andrea Scaloni, Valentina Pallottini, Susanna Iossa, and Luisa Cigliano. The first draft of the manuscript was written by Maria Stefania Spagnuolo and Luisa Cigliano, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data supporting the findings of this study are available within the article and its supplementary information files.

Declarations

Ethics Approval The study was conducted according to the guidelines of the Declaration of Helsinki, approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II and authorized by Italian Health Minister (4448/2019-PR).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

References

1. Jensen T, Abdelmalek MF, Sullivan S, Nadeau KJ, Green M, Roncal C, Nakagawa T, Kuwabara M et al (2018) Fructose and sugar: a major mediator of non-alcoholic fatty liver disease. J Hepatol 68(5):1063–1075. https://doi.org/10.1016/j.jhep.2018.01.019
2. Febbraio MA, Karin M (2021) "Sweet death": fructose as a metabolic toxin that targets the gut-liver axis. Cell Metab 33(12):2316–2328. https://doi.org/10.1016/j.cmet.2021.09.004
3. Ford CN, Slining MM, Popkin BM (2013) Trends in dietary intake among US 2- to 6-year-old children, 1989–2008. J Acad Nutr Diet 113(1):35–42. https://doi.org/10.1016/j.jand.2012.08.022
4. Albers AB, Siegel M, Ramirez RL, Ross C, DeJong W, Jernigan DH (2015) Flavored alcoholic beverage use, risky drinking behaviors, and adverse outcomes among underage drinkers: results from the ABRAND Study. Am J Public Health 105:810–815. https://doi.org/10.2105/AJPH.2014.3023
5. Hemmingsson E (2018) Early childhood obesity risk factors: socioeconomic adversity, family dysfunction, offspring distress, and junk food self-medication. Curr Obes Rep 7(2):204–209. https://doi.org/10.1007/s13679-018-0310-2
6. Perrar I, Schmitting S, Della Corte KW, Buyken AE, Alexy U (2020) Age and time trends in sugar intake among children and adolescents: results from the DONALD study. Eur J Nutr 59(3):1043–1054. https://doi.org/10.1007/s00394-019-01965-y
7. Elliott SS, Keim NL, Stern JS, Teff K, Havel PJ (2002) Fructose, weight gain, and the insulin resistance syndrome. Am J Clin Nutr 76(5):911–922. https://doi.org/10.1093/ajcn/76.5.911
8. Sun S, Anderson GH, Flickinger BD, Williamson-Hughes PS, Empie MW (2011) Fructose and non-fructose sugar intakes in the US population and their associations with indicators of metabolic syndrome. Food Chem Toxicol 49(11):2875–2882. https://doi.org/10.1016/j.fct.2011.07.068

9. Kit BK, Fakhouri TH, Park S, Nielsen SJ, Ogden CL (2013) Trends in sugar-sweetened beverage consumption among youth and adults in the United States: 1999–2010. Am J Clin Nutr 98(1):180–188. https://doi.org/10.3945/ajcn.112.057943
10. Meyers AM, Mourra D, Beeler JA (2017) High fructose corn syrup induces metabolic dysregulation and altered dopamine signaling in the absence of obesity. PLoS ONE 12(12):e0190206. https://doi.org/10.1371/journal.pone.0190206
11. Cohen JFW, Rifas-Shiman SL, Jessica Young J, Oken E (2018) Associations of prenatal and child sugar intake with child cognition. Am J Prev Med 54(6):727–735. https://doi.org/10.1016/j.amepre.2018.02.020
12. Noble EE, Hsu TM, Liang J, Kanoski SE (2019) Early-life sugar consumption has long-term negative effects on memory function in male rats. Nutr Neurosci 22(4):273–283. https://doi.org/10.1080/1028415X.2017.1378851
13. Spagnuolo MS, Iossa S, Cigliano L (2020) Sweet but bitter: focus on fructose impact on brain function in rodent models. Nutrients 13(1):1. https://doi.org/10.3390/nu13010001
14. Giedd JN, Blumenthal J, Jeffries NO, Castellanos FX, Liu H, Zijdenbos A, Paus T, Evans AC (1999) Brain development during childhood and adolescence: a longitudinal MRI study. Nat Neurosci 2(10):861–863. https://doi.org/10.1038/13158
15. Casey BJ, Jones RM, Hare TA (2008) The adolescent brain. Ann N Y Acad Sci 1124:111–126. https://doi.org/10.1196/annals.1440.010
16. Mills KL, Goddings AL, Clasen LS, Giedd JN, Blakemore SJ (2014) The developmental mismatch in structural brain maturation during adolescence. Dev Neurosci 36(3–4):147–160. https://doi.org/10.1159/000362328
17. Hensch TK (2005) Critical period plasticity in local cortical circuits. Nat Rev Neurosci 6:877–888. https://doi.org/10.1038/nrn1787
18. Lokken KL, Boeka AG, Austin HM, Gunstad J, Harmon CM (2009) Evidence of executive dysfunction in extremely obese adolescents: a pilot study. Surg Obes Relat Dis 5(5):547–552. https://doi.org/10.1016/j.soard.2009.05.008
19. Overby NC, Ludemann E, Hoigaard R (2013) Self-reported learning difficulties and dietary intake in Norwegian adolescents. Scand J Public Health 41(7):754–760. https://doi.org/10.1177/1403494813487449
20. Nyaradi A, Foster JK, Hickling S, Li J, Ambrosini GL, Jacques A, Oddy WH (2014) Prospective associations between dietary patterns and cognitive performance during adolescence. J Child Psychol Psychiatry 55(9):1017–1024. https://doi.org/10.1111/jcpp.12209
21. Nyaradi A, Oddy WH, Hickling S, Li J, Foster JK (2015) The relationship between nutrition in infancy and cognitive performance during adolescence. Front Nutr 2:2. https://doi.org/10.3389/fnut.2015.00002
22. Tau GZ, Peterson BS (2010) Normal development of brain circuits. Neuropsychopharmacology 35(1):147–168. https://doi.org/10.1038/npp.2009.115
23. Johnson RJ, Gomez-Pinilla F, Nagel M, Nakagawa T, Rodriguez-Iturbe B, Sanchez-Lozada LG, Tolan DR, Lanaspa MA (2020) Cerebral fructose metabolism as a potential mechanism driving Alzheimer's Disease. Front Aging Neurosci 12:560865. https://doi.org/10.3389/fnagi.2020.560865
24. Fierros-Campuzano J, Ballesteros-Zebadúa P, Manjarrez-Marmolejo J, Aguilera P, Méndez-Diaz M, Prospero-García O, Franco-Pérez J (2022) Irreversible hippocampal changes induced by high fructose diet in rats. Nutr Neurosci 25(6):1325–1337. https://doi.org/10.1080/1028415X.2020.1853418
25. Spagnuolo MS, Bergamo P, Crescenzo R, Iannotta L, Treppiccione L, Iossa S, Cigliano L (2020) Brain Nrf2 pathway, autophagy, and synaptic function proteins are modulated by a short-term fructose feeding in young and adult rats. Nutr Neurosci 23(4):309–320. https://doi.org/10.1080/1028415X.2018.1501532
26. Yin Q, Ma Y, Hong Y, Hou X, Chen J, Shen C, Sun M, Shang Y et al (2014) Lycopene attenuates insulin signaling deficits, oxidative stress, neuroinflammation, and cognitive impairment in fructose-drinking insulin resistant rats. Neuropharmacology 86:389–396. https://doi.org/10.1016/j.neuropharm.2014.07.020
27. Sangüesa G, Cascales M, Griñán C, Sánchez RM, Roglans N, Pallàs M, Laguna JC, Alegret M (2018) Impairment of novel object recognition memory and brain insulin signaling in fructose-but not glucose-drinking female rats. Mol Neurobiol 55(8):6984–6999. https://doi.org/10.1007/s12035-017-0863-1
28. Vilar M, Mira H (2016) Regulation of neurogenesis by neurotrophins during adulthood: expected and unexpected roles. Front Neurosci 10:26. https://doi.org/10.3389/fnins.2016.00026
29. Bekinschtein P, von Bohlen und Halbach O (2020) Editorial: cellular and molecular mechanisms of neurotrophin function in the nervous system. Front Cell Neurosci 14:101. https://doi.org/10.3389/fncel.2020.00101
30. Spijker S (2011) Dissection of rodent brain regions. In: Li K (ed) Neuroproteomics. Neuromethods 57:13-26. https://doi.org/10.1007/978-1-61779-111-6_2
31. Paxinos G, Watson C (1997) The Rat brain in stereotaxic coordinates. Academic Press, San Diego
32. Wasek B, Arning E, Bottiglieri T (2018) The use of microwave irradiation for quantitative analysis of neurotransmitters in the mouse brain. J Neurosci Methods 307:188–193. https://doi.org/10.1016/j.jneumeth.2018.05.016
33. Dienel GA (2021) Stop the rot. Enzyme inactivation at brain harvest prevents artifacts: a guide for preservation of the in vivo concentrations of brain constituents. J Neurochem 158(5):1007–1031. https://doi.org/10.1111/jnc.15293
34. Burtscher J, Zangrandi L, Schwarzer C, Gnaiger E (2015) Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues revealed by high- resolution respirometry. Mitochondrion 25:104–112. https://doi.org/10.1016/j.mito.2015.10.007
35. Spagnuolo MS, Pallottini V, Mazzoli A, Iannotta L, Tonini C, Morone B, Ståhlman M, Crescenzo R, Strazzullo M et al (2020) a short-term western diet impairs cholesterol homeostasis and key players of beta amyloid metabolism in brain of middle-aged rats. Mol Nutr Food Res 64(16):e2000541. https://doi.org/10.1002/mnfr.202000541
36. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1006/abio.1976.9999
37. Spagnuolo MS, Mollica MP, Maresca B, Cavaliere G, Cefaliello C, Trinchese G, Scudiero R, Crispino M et al (2015) High fat diet and inflammation - modulation of haptoglobin level in rat brain. Front Cell Neurosci 9:479. https://doi.org/10.3389/fncel.2015.00479
38. Spagnuolo MS, Donizetti A, Iannotta L, Aliperti V, Cupidi C, Bruni AC, Cigliano L (2018) Brain-derived neurotrophic factor modulates cholesterol homeostasis and Apolipoprotein E synthesis in human cell models of astrocytes and neurons. J Cell Physiol 233(9):6925–6943. https://doi.org/10.1002/jcp.26480
39. Spagnuolo MS, Maresca B, La Marca V, Carrizzo A, Veronesi C, Cupidi C, Piccoli T, Maletta RG et al (2014) Haptoglobin interacts with apolipoprotein E and beta-amyloid and influences their crosstalk. ACS Chem Neurosci 5(9):837–847. https://doi.org/10.1021/cn500099f
40. Mazzoli A, Spagnuolo MS, Gatto C, Nazzaro M, Cancelliere R, Crescenzo R, Iossa S, Cigliano L (2020) Adipose tissue and

brain metabolic responses to western diet-is there a similarity between the two? Int J Mol Sci 21(3):786. https://doi.org/10.3390/ijms21030786
41. Spagnuolo MS, Maresca B, Mollica MP, Cavaliere G, Cefaliello C, Trinchese G, Esposito MG, Scudiero R, Crispino M, Abrescia P et al (2014) Haptoglobin increases with age in rat hippocampus and modulates Apolipoprotein E mediated cholesterol trafficking in neuroblastoma cell lines. Front Cell Neurosci 8:212. https://doi.org/10.3389/fncel.2014.00212
42. Spagnuolo MS, Cigliano L, Abrescia P (2003) The binding of haptoglobin to apolipoprotein AI: influence of hemoglobin and concanavalin A. Biol Chem 384(12):1593–1596. https://doi.org/10.1515/BC.2003.176
43. Crescenzo R, Spagnuolo MS, Cancelliere R, Iannotta L, Mazzoli A, Gatto C, Iossa S, Cigliano L (2019) Effect of initial aging and high-fat/high-fructose diet on mitochondrial bioenergetics and oxidative status in rat brain. Mol Neurobiol 56(11):7651–7663. https://doi.org/10.1007/s12035-019-1617-z
44. Tipton KF, Davey G, Motherway M (2001) Monoamine oxidase assays. Curr Protoc Pharmacol Chapter 3(Unit3):6. https://doi.org/10.1002/0471141755.ph0306s09
45. Olesti E, Rodríguez-Morató J, Gomez-Gomez A, Ramaekers JG, de la Torre R, Pozo OJ (2019) Quantification of endogenous neurotransmitters and related compounds by liquid chromatography coupled to tandem mass spectrometry. Talanta 192:93–102. https://doi.org/10.1016/j.talanta.2018.09.034
46. Cigliano L, Spagnuolo MS, Abrescia P (2003) Quantitative variations of the isoforms in haptoglobin 1–2 and 2–2 individual phenotypes. Arch Biochem Biophys 416(2):227–237. https://doi.org/10.1016/s0003-9861(03)00318-7
47. Mazzoli A, Spagnuolo MS, Nazzaro M, Gatto C, Iossa S, Cigliano L (2021) Fructose removal from the diet reverses inflammation, mitochondrial dysfunction, and oxidative stress in hippocampus. Antioxidants (Basel) 10(3):487. https://doi.org/10.3390/antiox10030487
48. Bartesaghi S, Radi R (2018) Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration. Redox Biol 14:618–625. https://doi.org/10.1016/j.redox.2017.09.009
49. Haddad M, Perrotte M, Landri S, Lepage A, Fülöp T, Ramassamy C (2019) Circulating and extracellular vesicles levels of N-(1-Carboxymethyl)-L-Lysine (CML) differentiate early to moderate Alzheimer's Disease. J Alzheimers Dis 69(3):751–762. https://doi.org/10.3233/JAD-181272
50. Kowiański P, Lietzau G, Czuba E, Waśkow M, Steliga A, Moryś J (2018) BDNF: a key factor with multipotent impact on brain signaling and synaptic plasticity. Cell Mol Neurobiol 38(3):579–593. https://doi.org/10.1007/s10571-017-0510-4
51. Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: a major mediator of neuronal neurotrophin responses. Neuron 19(5):1031–1047. https://doi.org/10.1016/s0896-6273(00)80395-5
52. Alonso M, Vianna MR, Izquierdo I, Medina JH (2022) Signaling mechanisms mediating BDNF modulation of memory formation in vivo in the hippocampus. Cell Mol Neurobiol 22:663–674. https://doi.org/10.1023/a:1021848706159
53. Fahnestock M, Michalski B, Xu B, Coughlin M (2001) The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol Cell Neurosci 18(2):210–220. https://doi.org/10.1006/mcne.2001.1016
54. Fahnestock M, Shekari A (2019) ProNGF and neurodegeneration in Alzheimer's disease. Front Neurosci 13:129. https://doi.org/10.3389/fnins.2019.00129
55. Won S, Levy JM, Nicoll RA, Roche KW (2017) MAGUKs: multifaceted synaptic organizers. Curr Opin Neurobiol 43:94–101. https://doi.org/10.1016/j.conb.2017.01.006
56. Soreq H, Seidman S (2001) Acetylcholinesterase-new roles for an old actor. Nat Rev Neurosci 2(4):294–302. https://doi.org/10.1038/35067589
57. Yeung AWK, Georgieva MG, Atanasov AG, Tzvetkov NT (2019) Monoamine oxidases (MAOs) as privileged molecular targets in neuroscience: research literature analysis. Front Mol Neurosci 12:143. https://doi.org/10.3389/fnmol.2019.00143
58. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TW, Fiehn O et al (2007) Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics 3(3):211–221. https://doi.org/10.1007/s11306-007-0082-2
59. El Hamrani D, Gin H, Gallis JL, Bouzier-Sore AK, Beauvieux MC (2018) Consumption of alcopops during brain maturation period: higher impact of fructose than ethanol on brain metabolism. Front Nutr 5:33. https://doi.org/10.3389/fnut.2018.00033
60. Tognini P, Samad M, Kinouchi K, Liu Y, Helbling JC, Moisan MP, Eckel-Mahan KL, Baldi P et al (2020) Reshaping circadian metabolism in the suprachiasmatic nucleus and prefrontal cortex by nutritional challenge. Proc Natl Acad Sci U S A 17(47):29904–29913. https://doi.org/10.1073/pnas.2016589117
61. Dannenberg H, Young K, Hasselmo M (2017) Modulation of hippocampal circuits by muscarinic and nicotinic receptors. Front Neural Circuits 11:102. https://doi.org/10.3389/fncir.2017.00102
62. Conde Rojas I, Acosta-García J, Caballero-Florán RN, Jijón-Lorenzo R, Recillas-Morales S, Avalos-Fuentes JA, Paz-Bermúdez F, Leyva-Gómez G et al (2020) Dopamine D4 receptor modulates inhibitory transmission in pallido-pallidal terminals and regulates motor behavior. Eur J Neurosci 52(11):4563–4585. https://doi.org/10.1111/ejn.15020
63. Grogan JP, Sandhu TR, Hu MT, Manohar SG (2020) Dopamine promotes instrumental motivation, but reduces reward-related vigour. Elife 9:e58321. https://doi.org/10.7554/eLife.58321.sa2
64. Daubner SC, Le T, Wang S (2011) Tyrosine hydroxylase and regulation of dopamine synthesis. Arch Biochem Biophys 508(1):1–12. https://doi.org/10.1016/j.abb.2010.12.017
65. Gasiorowska A, Wydrych M, Drapich P, Zadrozny M, Steczkowska M, Niewiadomski W, Niewiadomska G (2021) The biology and pathobiology of glutamatergic, cholinergic, and dopaminergic signaling in the aging brain. Front Aging Neurosci 13:654931. https://doi.org/10.3389/fnagi.2021.654931
66. Mazzoli A, Gatto C, Crescenzo R, Cigliano L, Iossa S (2021) Prolonged changes in hepatic mitochondrial activity and insulin sensitivity by high fructose intake in adolescent rats. Nutrients 13(4):1370. https://doi.org/10.3390/nu13041370
67. Mazzoli A, Gatto C, Crescenzo R, Spagnuolo MS, Nazzaro M, Iossa S, Cigliano L (2021) Gut and liver metabolic responses to dietary fructose - are they reversible or persistent after switching to a healthy diet? Food Funct 12(16):7557–7568. https://doi.org/10.1039/d1fo00983d
68. Sautin YY, Nakagawa T, Zharikov S, Johnson RJ (2007) Adverse effects of the classic antioxidant uric acid in adipocytes: NADPH oxidase-mediated oxidative/nitrosative stress. Am J Physiol Cell Physiol 293(2):C584–C596. https://doi.org/10.1152/ajpcell.00600.2006
69. Yu MA, Sánchez-Lozada LG, Johnson RJ, Kang DH (2010) Oxidative stress with an activation of the renin-angiotensin system in human vascular endothelial cells as a novel mechanism of uric acid-induced endothelial dysfunction. J Hypertens 28(6):1234–1242
70. Lanaspa MA, Sanchez-Lozada LG, Choi YJ, Cicerchi C, Kanbay M, Roncal-Jimenez CA, Ishimoto T, Li N et al (2012) Uric acid induces hepatic steatosis by generation of mitochondrial oxidative stress: potential role in fructose-dependent and -independent fatty liver. J Biol Chem 287(48):40732–40744. https://doi.org/10.1074/jbc.M112.399899

71. Roberts RA, Smith RA, Safe S, Szabo C, Tjalkens RB, Robertson FM (2010) Toxicological and pathophysiological roles of reactive oxygen and nitrogen species. Toxicology 276(2):85–94. https://doi.org/10.1016/j.tox.2010.07.009
72. Goodfellow MJ, Borcar A, Proctor JL, Greco T, Rosenthal RE, Fiskum G (2020) Transcriptional activation of antioxidant gene expression by Nrf2 protects against mitochondrial dysfunction and neuronal death associated with acute and chronic neurodegeneration. Exp Neurol 328:113247. https://doi.org/10.1016/j.expneurol.2020.113247
73. Allaman I, Bélanger M, Magistretti PJ (2015) Methylglyoxal, the dark side of glycolysis. Front Neurosci 9:23. https://doi.org/10.3389/fnins.2015.00023
74. Akhter F, Chen D, Akhter A, Yan SF, Yan SS (2021) Age-dependent accumulation of dicarbonyls and advanced glycation endproducts (AGEs) associates with mitochondrial stress. Free Radic Biol Med 164:429–438. https://doi.org/10.1016/j.freeradbiomed.2020.12.021
75. Mastrocola R, Nigro D, Cento AS, Chiazza F, Collino M, Aragno M (2016) High-fructose intake as risk factor for neurodegeneration: key role for carboxy methyllysine accumulation in mice hippocampal neurons. Neurobiol Dis 89:65–75. https://doi.org/10.1016/j.nbd.2016.02.005
76. Cigliano L, Spagnuolo MS, Crescenzo R, Cancelliere R, Iannotta L, Mazzoli A, Liverini G, Iossa S (2018) Short-term fructose feeding induces inflammation and oxidative stress in the hippocampus of young and adult rats. Mol Neurobiol 55(4):2869–2883. https://doi.org/10.1007/s12035-017-0518-2
77. Schalkwijk CG, Stehouwer CDA (2020) Methylglyoxal, a highly reactive dicarbonyl compound, in diabetes, its vascular complications, and other age-related diseases. Physiol Rev 100(1):407–461. https://doi.org/10.1152/physrev.00001.2019
78. Harward SC, Hedrick NG, Hall CE, Parra-Bueno P, Milner TA, Pan E, Laviv T, Hempstead BL et al (2016) Autocrine BDNF-TrkB signalling within a single dendritic spine. Nature 538(7623):99–103. https://doi.org/10.1038/nature19766
79. Fahnestock M, Yu G, Michalski B, Mathew S, Colquhoun A, Ross GM, Coughlin MD (2004) The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. J Neurochem 89(3):581–592. https://doi.org/10.1111/j.1471-4159.2004.02360.x
80. Masoudi R, Ioannou M, Coughlin MD, Pagadala P, Neet KE, Clewes O, Allen SJ, Dawbarn D et al (2009) Biological activity of nerve growth factor precursor is dependent upon relative levels of its receptors. J Biol Chem 284(27):18424–18433. https://doi.org/10.1074/jbc.M109.007104
81. Moreira ELG, de Oliveira J, Nunes JC, Santos DB, Nunes FC, Vieira DS, Ribeiro-do-Valle RM, Pamplona FA et al (2012) Age-related cognitive decline in hypercholesterolemic LDL receptor knockout mice (LDLr−/−): evidence of antioxidant imbalance and increased acetylcholinesterase activity in the prefrontal cortex. J Alzheimers Dis 32(2):495–511. https://doi.org/10.3233/JAD-2012-120541
82. Moreira ELG, de Oliveira J, Engel DF, Walz R, de Bem AF, Farina M, Prediger RD (2014) Hypercholesterolemia induces short-term spatial memory impairments in mice: up-regulation of acetylcholinesterase activity as an early and causal event? J Neural Transm (Vienna) 121(4):415–426. https://doi.org/10.1007/s00702-013-1107-9
83. Ajayi AM, John KA, Emmanuel IB, Chidebe EO, Adedapo ADA (2021) High-fat diet-induced memory impairment and anxiety-like behavior in rats attenuated by peel extract of Ananas comosus fruit via atheroprotective, antioxidant and anti-inflammatory actions. Metabol Open 9:100077. https://doi.org/10.1016/j.metop.2021.100077
84. Cha SH, Wolfgang M, Tokutake Y, Chohnan S, Lane MD (2008) Differential effects of central fructose and glucose on hypothalamic malonyl-CoA and food intake. Proc Natl Acad Sci U S A 105(44):16871–18675. https://doi.org/10.1073/pnas.0809255105
85. Macedo-Lima M, Remage-Healey L (2021) Dopamine modulation of motor and sensory cortical plasticity among vertebrates. Integr Comp Biol 61(1):316–336. https://doi.org/10.1093/icb/icab019
86. Beeler JA, Faust RP, Turkson S, Ye H, Zhuang X (2016) Low dopamine D2 receptor increases vulnerability to obesity via reduced physical activity, not increased appetitive motivation. Biol Psychiatry 79(11):887–897. https://doi.org/10.1016/j.biopsych.2015.07.009
87. Friend DM, Devarakonda K, O'Neal TJ, Skirzewski M, Papazoglou I, Kaplan AR, Liow JS, Guo J et al (2017) Basal ganglia dysfunction contributes to physical inactivity in obesity. Cell Metab 25(2):312–321. https://doi.org/10.1016/j.cmet.2016.12.001
88. De Souza L, Barros WM, De Souza RM, Delanogare E, Machado AE, Braga SP, Rosa GK, Nardi GM et al (2021) Impact of different fructose concentrations on metabolic and behavioral parameters of male and female mice. Physiol Behav 228:113187. https://doi.org/10.1016/j.physbeh.2020.113187
89. Kloster A, Hyer MM, Dyer S, Salome-Sanchez C, Neigh GN (2021) High fructose diet induces sex-specific modifications in synaptic respiration and affective-like behaviors in rats. Neuroscience 454:40–50. https://doi.org/10.1016/j.neuroscience.2019.11.039

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Primary and secondary antibodies dilutions used for Western blotting analysis

Antigen	Primary Antibody	Secondary Antibody
Glut-5	Invitrogen (PA5-80023); 1:1,000[a]	GAR-HRP IgG; 1:45,000[b]
Glut-4	Santa Cruz Biotechnology (sc-53566); 1:1,000[a]	GAM-HRP IgG; 1:15,000[b]
GFAP	Cell Signalling Technology (#12389); 1:1,000[c]	GAR-HRP IgG; :150,000[d]
Synaptophysin	AB9272; Merk-Millipore; 1:150,000[a]	GAR-HRP IgG; 1:40,000[e]
Synaptotagmin I	Cell Signalling Technology (#14588); 1:1,000[c]	GAR-HRP IgG; :200,000[f]
PSD-95	Cell Signalling Technology (#2507); 1:1,000[c]	GAR-HRP IgG; 1:70,000[f]
BDNF	Santa Cruz Biotechnology (sc-546); 1:500[c]	GAR-HRP IgG; 1:70,000[d]
pro-BDNF	Santa Cruz Biotechnology (sc-546); 1:1,000[c]	GAR-HRP IgG; 1:10,000[d]
PGC-1α	Merk-Millipore (#AB3242); 1:2,000[a]	GAR-HRP IgG; 1:20,000[b]
TrkB	Santa Cruz Biotechnology (sc-3772218); 1:2,000[a]	GAR-HRP IgG; :140,000[b]
pErk1/2	Cell Signalling Technology (#9101S); 1:1,000[a]	GAR-HRP IgG; :150,000[b]
Erk1/2	Cell Signalling Technology (#9102); 1:1,000[a]	GAR-HRP IgG; :170,000[b]
TH	Santa Cruz Biotechnology (sc-25269); 1:1,000[d]	GAM-HRP IgG; 1:10,000[d]
TrkA	Santa Cruz Biotechnology (sc-118); 1:1,000[d]	GAR-HRP IgG; 1:10,000 [d]
p75NTR	Santa Cruz Biotechnology (sc-271708); 1:1,000[d]	GAM-HRP IgG; 1:10,000[d]
NGF	Santa Cruz Biotechnology (sc-365944); 1:1,000[d]	GAM-HRP IgG; 1:10,000[d]
β -Actin	Sigma-Aldrich (A2228); 1:1,000[c]	GAM-HRP IgG; 1:35,000[e]
Vinculin	Sigma-Aldrich (V9131); 1:10,000[d]	GAM-HRP IgG; 1:10,000[d]

GAR-HRP: Goat anti-rabbit Horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA; catalogue number GtxRb-003-DHRPX); **GAM-HRP**: Goat anti-mouse Horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA; catalogue number GtxMu-003-DHRPX); **T-TBS**: 130 mM NaCl, 20 mM Tris-HCl, 0.05% Tween, pH 7.4; [a] T-TBS containing 3% w/v BSA; [b]: T-TBS containing 2% w/v BSA; [c]: T-TBS containing 3% v/v non-fat milk; [d]: T-TBS containing 5% v/v non-fat milk; [e]: T-TBS containing 1% v/v non-fat milk; [f]: T-TBS containing 3%v/vnon-fatmilk.

Supplementary Table 2. Differentially over-represented compounds as determined by HILIC-Fourier transform mass spectrometry (FTMS) untargeted analysis in control adolescent (C) and fructose-fed adolescent (F) rats. Error (Δ ppm) was calculated as the ratio between the difference of the theoretical mass minus the experimental mass and the theoretical mass, multiplied per one million. RT, retention time; metabolite identification level was defined according to Metabolomics Standards Initiative (MSI) (Sumner et al., 2007); calc. MW, calculated molecular weight.

Name	Formula	Δ [ppm]	Calc. MW	RT [min]	Ratio: (F) / (C)	Log2 Fold Change: (F) / (C)	P-value: (F) / (C)	MSI level
Creatine	C4 H9 N3 O2	-3.39	131.06903	7.209	1.788	0.84	1.55986E-06	2
N,N-Dimethylglycine	C4 H9 N O2	-4.74	103.06284	6.477	1.464	0.55	0.001806643	2
N-Acetyl-L-aspartic acid	C6 H9 N O5	-2.26	175.04768	7.409	1.95	0.96	1.1877E-05	2
Arabinosylhypoxanthine	C10 H12 N4 O5	-1.92	268.08026	6.635	1.487	0.57	0.000377137	2
DL-Phenylalanine	C9 H11 N O2	-1.91	165.07866	7.203	1.758	0.81	0.013029206	2
L-(+)-Valine	C5 H11 N O2	-3.32	117.07859	7.32	1.783	0.83	0.00138459	2
	C2 H8 N3 O4 P	-1.11	169.02505	7.188	1.553	0.64	0.000743623	4
L-(-)-Methionine	C5 H11 N O2 S	-2.84	149.05063	7.334	2.024	1.02	0.009802015	2
L-(+)-Alanine	C3 H7 N O2	-4.46	89.04728	7.593	1.819	0.86	1.14045E-09	2
L-Tyrosine	C9 H11 N O3	-2.08	181.07352	7.348	1.731	0.79	1.60125E-05	2
	C10 H6 N6 O6	4.36	306.03621	6.622	1.568	0.65	0.000444015	4
Guanosine	C10 H13 N5 O5	-1.58	283.09122	7.453	2.404	1.27	1.14514E-05	2
L-Proline	C5 H9 N O2	-3.74	115.0629	7.954	32.442	5.02	0.000354262	2
	C7 H3 N5 P2 S	3.87	250.95941	7.423	2.698	1.43	7.84582E-07	4
DL-Arginine	C6 H14 N4 O2	-1.82	174.11136	7.308	1.45	0.54	0.002900577	2
	C7 H10 N6 O7	3.97	290.06225	6.616	1.571	0.65	0.013255	4
L-Lysine	C6 H14 N2 O2	-2.75	146.10513	7.39	1.884	0.91	0.001095626	2
L-Tryptophan	C11 H12 N2 O2	-1.04	204.08967	7.197	1.922	0.94	0.000201327	2
	C8 H N3 O6	-3.8	234.98564	7.419	2.115	1.08	3.0846E-05	4
	C11 H8 N2 O10	0.17	328.01795	6.633	1.462	0.55	0.010055157	4

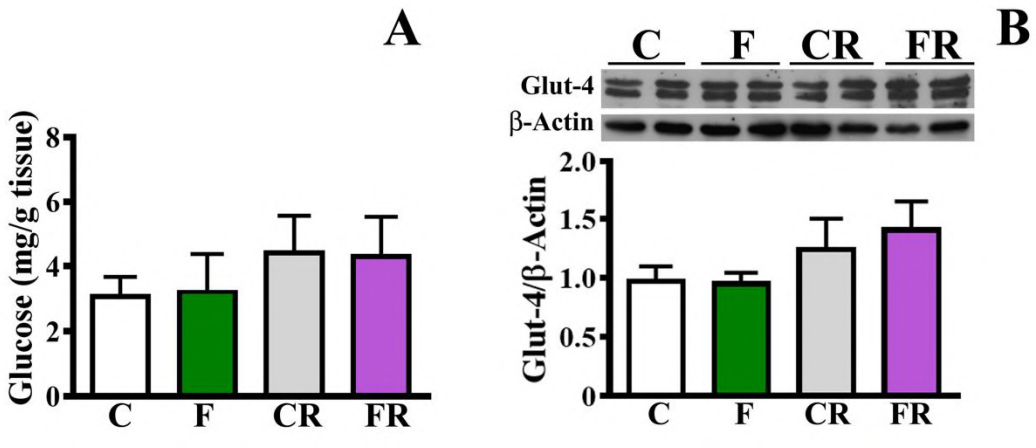
Supplementary Table 3. Differentially over-represented compounds as determined by HILIC-Fourier transform mass spectrometry (FTMS) untargeted analysis in young-adult control rescued (CR) and young-adult fructose-rescued (FR) rats. Error (Δ ppm) was calculated as the ratio between the difference of the theoretical mass minus the experimental mass and the theoretical mass, multiplied per one million. RT, retention time; metabolite identification level was defined according to Metabolomics Standards Initiative (MSI) (Sumner et al., 2007); calc. MW, calculated molecular weight.

Name	Formula	Δ [ppm]	Calc. MW	RT [min]	Ratio: (FR) / (CR)	Log2 Fold Change: (FR) / (CR)	P-value: (FR) / (CR)	MSI level
L-Glutamic acid	C5 H9 N O4	-3.07	147.05271	8.462	1.507	0.59	7.10273E-07	2
N-Acetyl-L-aspartic acid	C6 H9 N O5	-1.7	175.04777	7.387	1.945	0.96	2.69033E-05	2
1-valeryl-pyrrolidine,	C9 H17 N O	-3.94	155.1304	2.352	76.734	6.26	2.59576E-15	2
n-Hexanamide	C6 H13 N O	-2.68	115.09941	2.268	1.521	0.61	0.007465124	2
	C13 H35 N6 P3 S2	3.1	432.15908	8.463	1.531	0.61	7.8472E-06	4
6-[(6-Aminohexanoyl)amino]hexanoic acid	C12 H24 N2 O3	-0.86	244.17848	3.098	19.622	4.29	3.21218E-07	2
L-Lysine	C6 H14 N2 O2	-2.04	146.10523	7.401	2.332	1.22	2.29768E-05	2
Indole derivative	C16 H23 N5 O	-4.67	301.18885	2.701	56.171	5.81	3.13247E-05	2
CEL	C9 H18 N2 O4	-0.17	218.12662	7.013	2.359	1.24	0.001400264	2
	C8 H15 N2 O5 P3 S	0.79	343.99172	6.665	1.532	0.62	0.000199173	4
L-homoserine lactone	C4 H7 N O2	-3.36	101.04734	8.465	1.498	0.58	3.76852E-06	2
	C16 H39 N9 O2 P2	-0.01	451.27019	3.813	1.508	0.59	0.01782836	4
	C14 H29 N3 O2	-0.7	271.22579	4.475	1.563	0.64	0.018627875	4
	C9 H21 N O2	-0.19	175.1572	3.363	1.987	0.99	0.000869172	4
N-pentanoylphenylalanine	C14 H19 N O3	-0.41	249.13639	3.334	2.207	1.14	9.66955E-06	2
	C16 H42 N5 P3	4.61	397.26714	2.165	1.441	0.53	0.011179231	4
	C18 H47 N9 O2 P2 S	3.18	515.30651	2.248	2.544	1.35	0.000628253	4
	C12 H22 N2 O2	-0.05	226.16812	3.424	2.933	1.55	0.000433627	4

Supplementary Table 4. Targeted zwitterionic HILIC-Fourier transform mass spectrometry (FTMS) analysis. Error (Δ ppm) was calculated as the ratio between the difference of the theoretical mass minus the experimental mass and the theoretical mass, multiplied per one million. RT, retention time; GABA, γ-aminobutyric acid. Metabolite identification level, as defined according to Metabolomics Standards Initiative (MSI) (Sumner et al., 2007), was 1 for all the listed compounds upon matching with authentic reference analytical standards.

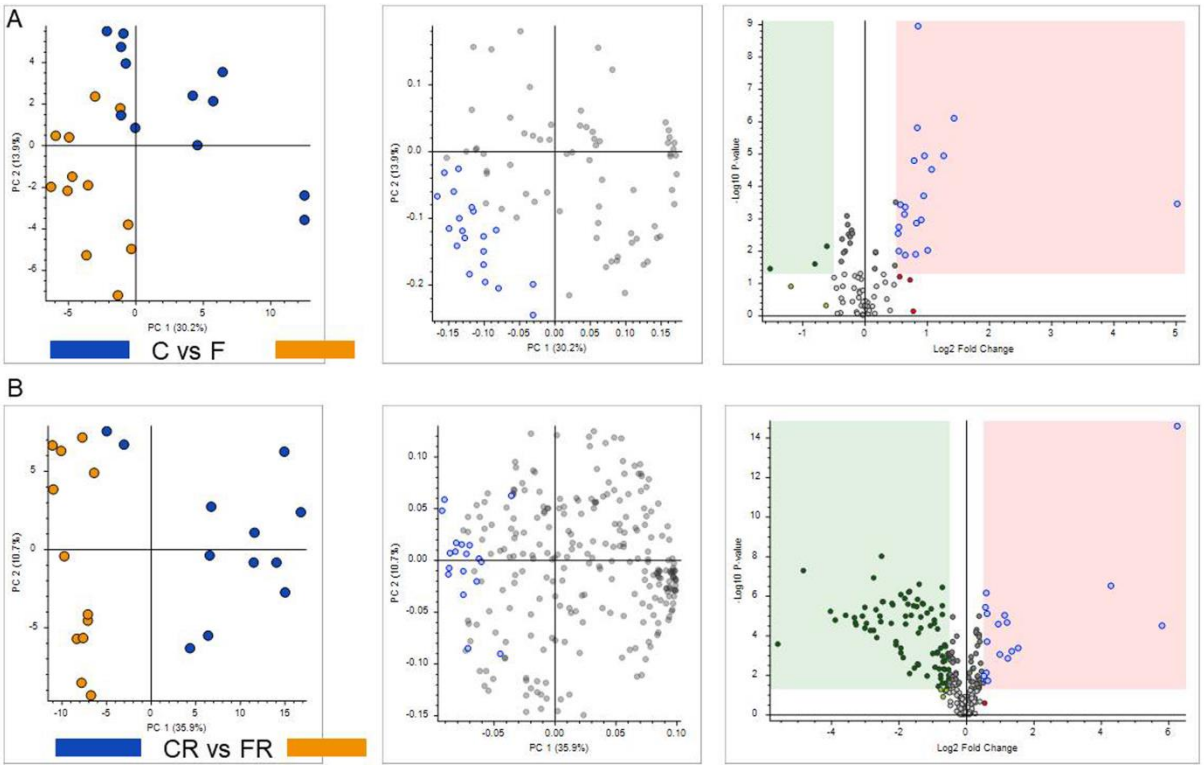
Compound Name	**RT**	**Elemental composition**	***m/z theoretical***	***m/z experimental***	Δ ppm
Nε-carboxymethyllysine	7.2	$C_8H_{16}N_2O_4$	205.11832	205.11801	-1.5
Nε-carboxyethyllysine	7.5	$C_9H_{18}N_2O_4$	219.13393	219.13407	0.6
Glutamate	8.5	$C_5H_9NO_4$	148.06043	148.06085	2.8
GABA	5.7	$C_4H_9NO_2$	104.07060	104.07051	-0.9
Tyrosine	7.4	$C_9H_{11}NO_3$	182.08117	182.08132	0.8
Tyramine	6.7	$C_8H_{11}NO$	138.09134	138.09117	-1.2
Dopamine	3.5	$C_8H_{11}NO_2$	154.08625	154.08658	2.1
Acetylcholine	4.9	$C_7H_{16}NO_2^+$	146.11755	146.11781	1.8

Supplementary Figure 1. Evaluation of Glucose and Glut-4 in rat frontal cortex



(a) Glucose amount; b) Glut-4 level (representative western blot and densitometric analysis) in frontal cortex of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Data are the means ± SD of 8 rats/group.

Supplementary Figure 2. Principal component analysis (PCA), loadings plot and volcano plot. Panel A reports explorative PCA with control adolescent (C) and fructose-fed adolescent (F) rats, along with loadings plot and volcano plot based on differntial analysis with a p-value 0.05, while for log2 fold change 0.5 was used. All the metabolites reported in Supplementary Table 2 are shown in light blue. Panel B reports the PCA, loadings and volcano plots from young-adult control rescued (CR) and young-adult fructose-rescued (FR) rats. Light blue spotted points depict differentially over-represented metabolites listed in Supplementary Table 3. Due to the low number of metabolites down-represented in C vs F volcano plot (panel A), we only focused on over-represented metabolites.



Chapter 4

Fructose diet-associated molecular alterations in hypothalamus of adolescent rats: a proteomic approach

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Fructose Diet-Associated Molecular Alterations in Hypothalamus of Adolescent Rats: A Proteomic Approach

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Abstract: Background: The enhanced consumption of fructose as added sugar represents a major health concern. Due to the complexity and multiplicity of hypothalamic functions, we aim to point out early molecular alterations triggered by a sugar-rich diet throughout adolescence, and to verify their persistence until the young adulthood phase. Methods: Thirty days old rats received a high-fructose or control diet for 3 weeks. At the end of the experimental period, treated animals were switched to the control diet for further 3 weeks, and then analyzed in comparison with those that were fed the control diet for the entire experimental period. Results: Quantitative proteomics identified 19 differentially represented proteins, between control and fructose-fed groups, belonging to intermediate filament cytoskeleton, neurofilament, pore complex and mitochondrial respiratory chain complexes. Western blotting analysis confirmed proteomic data, evidencing a decreased abundance of mitochondrial respiratory complexes and voltage-dependent anion channel 1, the coregulator of mitochondrial biogenesis PGC-1α, and the protein subunit of neurofilaments α-internexin in fructose-fed rats. Diet-associated hypothalamic inflammation was also detected. Finally, the amount of brain-derived neurotrophic factor and its high-affinity receptor TrkB, as well as of synaptophysin, synaptotagmin, and post-synaptic protein PSD-95 was reduced in sugar-fed rats. Notably, deregulated levels of all proteins were fully rescued after switching to the control diet. Conclusions: A short-term fructose-rich diet in adolescent rats induces hypothalamic inflammation and highly affects mitochondrial and cytoskeletal compartments, as well as the level of specific markers of brain function; above-reported effects are reverted after switching animals to the control diet.

Keywords: proteomics; hypothalamus; fructose-rich diet; adolescence; mitochondria; inflammation

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1. Introduction

The crucial role of nutrition for cerebral health and the impact of dietary habits on brain structure and function has been long and far recognized [1–3]. To date, the increased consumption of fructose as added sugar in many types of drinks and processed foods [4,5], especially among young people [6], represents a major health concern. High fructose intake has been pointed out as the possible culprit for the raised incidence of chronic pathologies [7–10], and fructose over-consumption was shown to be associated with the over-activation of its cerebral metabolism [4], which was proposed to negatively impact on the whole brain physiology and cognitive function [4,11]. Notably, we previously reported that a short-term fructose-rich diet induces hippocampal mitochondrial dysfunction, oxidative stress, and neuroinflammation in young rats [12,13], as well as the imbalance of redox status, autophagic mechanisms and synaptic markers in the frontal cortex of both adult and young rats [14]. Further, studies in animal models have revealed that high-fructose diets impair hippocampal functions during childhood and adolescence [15–19], which represent critical phases of neurodevelopment.

The hypothalamus plays a key role in maintaining whole-body homeostasis [20,21]. Long-term fructose overfeeding was reported to alter the hypothalamic-pituitary-adrenal axis, thus causing glucocorticoid increase in peri-adolescent rats [22]. Further, fructose overconsumption was associated with impairment of hypothalamic insulin signaling [23,24], oxidative stress [24,25], and inflammation [23,26,27]; it was proposed that fructose-driven perturbations of hypothalamic function may alter the potential for satiety, thereby enhancing the possibility of developing obesity [28].

Data currently available on hypothalamic dysfunctions related to a high-fructose diet essentially refer to the effects of long-term sugar feeding, while information on corresponding alterations associated with short-term dietary treatment, particularly in the critical period of adolescence, is still lacking. Due to the complexity and multiplicity of hypothalamic functions, there is also the need for a holistic characterization aimed at unveiling the general picture of hypothalamic dysfunctions associated with a high-fructose diet. To fill this gap, we investigated adolescent rats fed a fructose-rich or control diet, for 3 weeks. To verify whether the fructose-driven changes persist after the switch to a control diet, half of the rats from both animal groups were then assigned to the control diet for additional 3 weeks until the young adulthood phase. Quantitative proteomics on hypothalamic extracts of all animal groups was used to identify molecular alterations triggered by a fructose-rich diet and to obtain insights into the relationship between sugar feeding and possible dysfunctions of the hypothalamus.

2. Materials and Methods

2.1. Materials

Bovine serum albumin fraction V (BSA), prestained protein ladder, salts, and buffers were purchased from DelTek (Naples, Italy). Rabbit anti-human haptoglobin IgG was from Sigma-Aldrich (St. Louis, MO, USA). Fuji Super RX 100 film, developer, and fixer were purchased by Laboratorio Elettronico di Precisione (Naples, Italy). BCA Protein assay kit, TMT Label Reagent Set, Pierce ™ High pH Reversed-Phase Peptide fractionation kit, Acclaim PepMap TM RSLC C18 column were from Thermo-Fisher Scientific (Waltham, MI, USA).

2.2. Experimental Design

Just weaned male Wistar rats (30 days old; Charles River; Calco, Como, Italy) were used. Housing, treatments, and euthanasia of animals were carried out as previously reported [13]. The rats were randomly assigned to two experimental groups, one fed a fructose-rich diet (F group; N = 16), and the other one fed a control diet (C group; N = 16) for 3 weeks. At the end of treatment, half of the rats from each group (C = 8; F = 8; 51 days old) were euthanized. Sugar-fed and control-fed rats were then assigned to the control diet for additional 3 weeks (FR group, N = 8; CR group, N = 8; respectively) until the young adulthood phase (72 days old). The experimental design is reported in Figure 1.

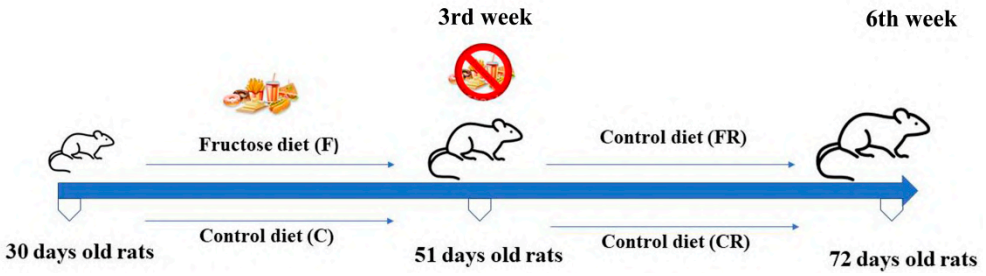

Figure 1. Experimental design.

Thirty days-old rats (just weaned animals) received a control (C; N = 16) or fructose-rich (F; N = 16) diet for three weeks, until the adolescence phase (51 days old rats). In the 3rd week eight rats from each group were euthanized. Eight animals of both fructose-fed

and control groups were fed the control diet (FR, N = 8; CR, N = 8) for further three weeks, until young adulthood (6th week).

The control and the fructose-rich diet were isocaloric, as they differ only in terms of the qualitative content of carbohydrates. Indeed, as shown in Table 1, the control diet contains starch instead of fructose. The here used control diet can be considered a glucose-containing diet, as starch digestion to glucose is very rapid [29,30].

Table 1. Nutritional composition of diets.

Ingredients (g/100 g)	Control Diet	Fructose Diet
Standard Chow [a]	50.5	50.5
Sunflower Oil	1.5	1.5
Casein	9.2	9.2
Alphacel	9.8	9.8
Cornstarch	20.4	-
Fructose	-	20.4
Water	6.4	6.4
AIN-76 mineral mix	1.6	1.6
AIN-76 vitamin mix	0.4	0.4
Choline	0.1	0.1
Methionine	0.1	0.1
Energy content and composition		
Gross Energy Density (kJ/g)	17.2	17.2
ME content (kJ/g) [b]	11.1	11.1
Proteins (% ME)	29.0	29.0
Lipids (% ME)	10.6	10.6
Carbohydrates (% ME)	60.4	60.4
Of which:		
Fructose	-	30.0
Starch	52.8	22.8
Sugars	7.6	7.6

[a] 4RF21, Mucedola, Italy. [b] Estimated by computation using the following values (kJ/g) for energy content: proteins = 16.736, lipids = 37.656, carbohydrates = 16.736. ME = metabolizable energy.

Animals were then euthanized, brains were removed and placed on an ice-cooled glass plate putting the face down the cortex. For dissecting the hypothalamus from each brain incisions were made along these margins: laterally 2 mm of the third ventricle, dorsally 2 mm starting from the base, rostro caudally from the optic chiasm to the posterior side of the mammillary bodies. Samples were then snap-frozen in liquid nitrogen and stored at −80 °C for further analyses.

2.3. Protein Extraction

Proteins were extracted by homogenizing aliquots of the hypothalamus (about 40 mg) in six volumes (*w*/*v*) of cold RIPA buffer [31]. The protein concentration of each homogenate was evaluated spectrophotometrically, using a commercial colorimetric kit (Bio-Rad, Hercules, CA, USA). Protein extracts were used for proteomic investigations, ELISA, and western blotting analysis.

2.4. Proteomic Analysis

For quantitative proteomic analysis, the protein concentration of pooled samples was quantified with the BCA Protein assay kit, as specified by the manufacturer. Peptide preparation was carried out as previously reported [32]. The resulting peptides from each protein sample were tagged with the TMT Label Reagent Set according to the matching C-TMT6-126, CR-TMT6-127, F-TMT6-128, FR-TMT6-129, at 25 °C, in agreement to manufacturer's instructions.

The analysis of TMT-labelled peptide fractions was carried out in triplicate on a nanoLC-ESI-Q-Orbitrap-MS/MS platform (Thermo Fisher Scientific, Waltham, MI, USA), as previously published [32].

All MS and MS/MS raw data files per sample were merged for protein identification and relative protein quantification into ProteomeDiscoverer 2.4 software (Thermo Scientific), enabling the database search by Mascot algorithm v. 2.4.2 (Matrix Science, London, UK) using the following criteria: UniProtKB protein database (*Rattus norvegicus*, 36206 protein sequences 11/2021). The mass spectrometry-based proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD038834.

Functional enrichment analysis including GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was performed by using EnrichR (https://amp.pharm.mssm.edu/Enrichr, accessed on 1 December 2022) and FunRich 3.1.3 (http://www.funrich.org, accessed on 25 November 2022). STRING (https://string-db.org, accessed on 5 August 2022) was used to visualize and integrate complex networks of proteomic data.

2.5. Western Blotting

Hypothalamic proteins (30 μg) were fractionated by denaturing and reducing electrophoresis [33] by 12.5% (to titrate glial fibrillary acidic protein (GFAP), synaptophysin, brain-derived neurotrophic factor (BDNF), voltage-dependent anion-selective channel 1 (VDAC-1), PTEN-induced putative kinase 1 (PINK-1), adiponectin, respiratory mitochondrial complexes I-V (OXPHOS)) or 10% (nuclear factor kappa-light-chain-enhancer of activated B cells, NFkB; post-synaptic density protein 95 (PSD-95), synaptotagmin I, peroxisome proliferator-activated receptor gamma coactivator 1-alpha [PGC-1α], tropomyosin receptor kinase B (TrkB), α-internexin) polyacrylamide gels. Protein blotting onto PVDF or nitrocellulose membrane (GE Healthcare; Milan, Italy), washing, and blocking steps were carried out according to previously published procedures [12,34].

The membranes were treated with primary antibodies (overnight, at 4 °C), and then incubated (1 h, at 37 °C) with the appropriate peroxidase-conjugated secondary antibodies. The specific dilution of each antibody is shown in Table 2. In particular, as the accurate quantification of each mitochondrial complex requires the use of different dilutions of secondary antibody for optimizing band intensities [35], it was used GAM-HRP IgG diluted 1:100,000 for the detection of complex I, 1:80,000 for complex II, 1:70,000 for complex IV, and 1:200,000 for complexes V and II.

After detection of each antigen, the membranes were stripped [36] and incubated (overnight, 4 °C) with mouse anti-β-actin IgG (1:1000 in 0.25% *v*/*v* non-fat milk) followed by GAM-HRP IgG (1:30,000 in 0.25% *v*/*v* non-fat milk; 1 h, 37 °C), in order to reveal β-actin, which was used as a loading control. Signal detection was carried out using the Excellent Chemiluminescent Kit Westar Antares (Cyanagen s.r.l., Bologna, Italy). Densitometric analysis of chemidoc or digital images of X-ray films exposed to immunostained membranes was performed with Un-Scan-It gel software (Silk Scientific, Orem, UT, USA).

Table 2. Dilutions of primary and secondary antibodies used for Western blotting.

	Primary Antibody	Secondary Antibody
GFAP	Cell Signalling Technology; 1:1000 [a]	GAR-HRP IgG; :100,000 [f]
Synaptophysin	Merk-Millipore; 1:100,000 [b]	GAR-HRP IgG; 1:35,000 [a]
Synaptotagmin I	Cell Signalling Technology; 1:1000 [c]	GAR-HRP IgG; :200,000 [c]
PSD-95	Cell Signalling Technology; 1:1000 [c]	GAR-HRP IgG; 1:60,000 [c]
BDNF	Abcam, Cambridge, UK (EPR1292); 1:2000 [d]	GAR-HRP IgG; 1:180,000 [a]
PGC-1α	Merk-Millipore; 1:2000 [b]	GAR-HRP IgG; 1:40,000 [b]
TrkB	Santa Cruz Biotechnology; 1:2000 [d]	GAR-HRP IgG; :100,000 [d]
VDAC 1	Santa Cruz Biotechnology; 1:500 [d]	GAM-HRP IgG; :50,000 [b]
α-internexin	Santa Cruz Biotechnology; 1:500 [d]	GAM-HRP IgG; :70,000 [b]
PINK1	Santa Cruz Biotechnology; 1:500 [d]	GAM-HRP IgG; 1:40,000 [b]
OXPHOS	Abcam, Cambridge, UK; 1:400 [b]	GAM-HRP IgG; 1:70,000–1:200,000 [a]
pNFkB	Santa Cruz Biotechnology; 1:200 [d]	GAM-HRP IgG; 1:50,000 [b]
NFkB	Santa Cruz Biotechnology; 1:500 [b]	GAM-HRP IgG; 1:15,000 [b]
β-Actin	Sigma-Aldrich; 1:1000 [e]	GAM-HRP IgG; 1:30,000 [e]

GAR-HRP: Goat anti-rabbit Horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA). GAM-HRP: Goat anti-mouse Horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA). T-TBS: 130 mM NaCl, 20 mM Tris-HCl, 0.05% Tween, pH 7.4; [a] T-TBS containing 1% *v*/*v* non-fat milk; [b] T-TBS containing 3% *w*/*v* BSA; [c] T-TBS containing 3% *v*/*v* non-fat milk; [d] T-TBS containing 2% *w*/*v* BSA; [e] T-TBS containing 0.25% *v*/*v* non-fat milk.

2.6. Analysis of Tumor Necrosis Factor Alpha (TNF-alpha) and Interleukin 6 (IL-6)

TNF-alpha and IL-6 concentrations were evaluated by sandwich ELISA with the DuoSet ELISA kit (R&D, DBA Italia, Segrate, MI, Italy), following the manufacturer's instructions. Hypothalamic homogenates were diluted 1:25 in the assay [37], and data are expressed as pg per mg of total proteins.

2.7. Evaluation of Nitro-Tyrosine and Haptoglobin (Hpt)

Nitro-tyrosine (N-Tyr) concentration was measured by ELISA in hypothalamic samples diluted 1:1500, 1:3000, and 1:6000 with coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6), essentially according to a previously published procedure [13]. Results are expressed as OD per mg of total proteins.

Haptoglobin (Hpt) was titrated by ELISA, in samples diluted 1: 2000, 1:8000, 1:25,000 with coating buffer, according to [38].

2.8. Statistical Analysis

Data are reported as mean values ± SEM. Normal distribution of data was verified with the GraphPad Prism 9.3.1 program (GraphPad Software, San Diego, CA, USA); the same software was used to perform one-way ANOVA followed by Bonferroni post-test. $p < 0.05$ was assumed as significant in the reported analyses.

3. Results

3.1. Identification of Fructose-Induced Hypothalamic Changes by Proteomic Analysis

Quantitative protein evaluation performed in the four experimental groups according to a Tandem Mass Tag (TMT)-based proteomic approach [39], followed by bioinformatic analysis, allowed the identification and quantification of 2521 unique protein entries in rat hypothalamus. Based on precise and accurate quantitation characteristics of the TMT-based proteomic approach, as determined in previous comparative studies on label-free and label-based procedures [40,41], only proteins with a concomitant fold change value > 1.2 and an abundance ratio *p*-value < 0.05 were considered as differentially represented. Accordingly, three proteins resulted to be over-represented, and sixteen were under-represented in the F group, with respect to the C group (Figure 2A, Supplementary Materials Figures S1 and S2); on the other hand, four proteins were over-represented in the FR group with respect to CR (Figure 2A; Supplementary Materials Figure S2). Slight protein quantitative

changes observed in F compared to the FR group, and in C compared to the CR group, were associated with youth to adolescence physiological changes, and thus were not considered in this study. Detailed quantitative proteomic data are reported in Supplementary Materials Table S1.

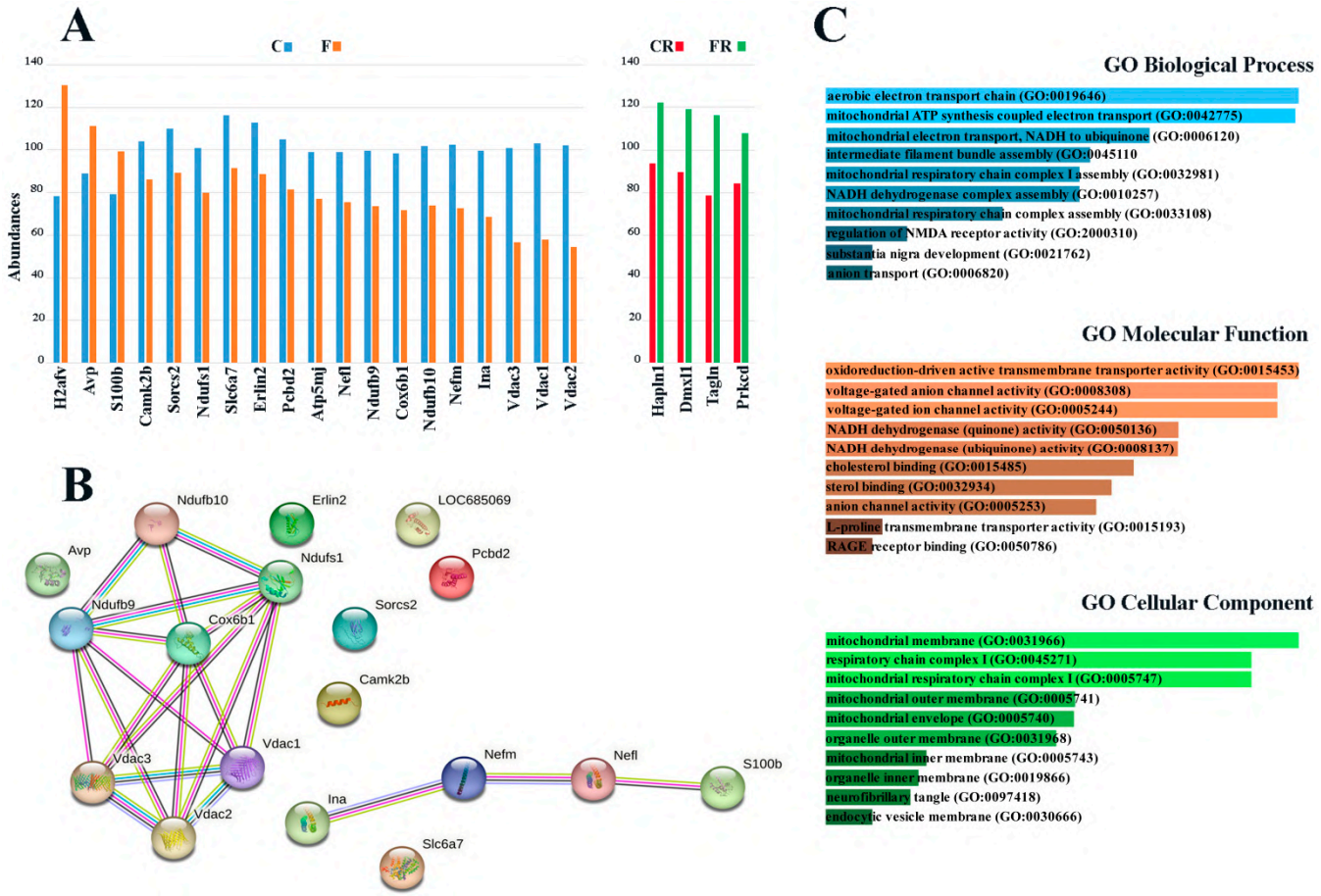

Figure 2. Proteomic analysis of rat hypothalamus. (**A**) Proteins showing quantitative changes in the comparison of fructose-fed adolescent (F, orange) versus control adolescent (C, light blue) rats, and young-adult fructose-rescued (FR, green) versus young-adult control rescued (CR, red) rats. Differentially represented proteins are indicated using corresponding gene names. (**B**) STRING analysis of differentially represented proteins present in F versus C fed rats. Functional protein associations were based on data recorded for *R. norvegicus*. Only medium-confidence interactions (0.4) are shown. Sorcs2, sortilin-related VPS10 domain containing receptor 2; Ndufs1, NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial; Atp5mpl (Atp5mj), mitochondrial membrane ATP synthase F (1) F (0); Camk2b, calcium/calmodulin-dependent protein kinase type II, subunit beta; S100b, protein S100-B; Ndufb10, NADH:ubiquinone oxidoreductase subunit B10; Vdac2, voltage-dependent anion-selective channel protein 2; Vdac1, voltage-dependent anion-selective channel protein 1; Ndufb9, NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9; Slc6a7, solute carrier family 6 (neurotransmitter transporter, l-proline) member 7; Avp, vasopressin-neurophysin 2-copeptin; Nefl, neurofilament, light polypeptide; Erlin2, ER lipid raft associated 2; Cox6b1, cytochrome c oxidase, subunit VIb polypeptide 1; Vdac3, voltage-dependent anion-selective channel protein 3; LOC685069, H2A histone family, member V; Ina, internexin neuronal intermediate filament protein, alpha; Nefm, neurofilament, medium polypeptide; Pcbd2, 4a-hydroxytetrahydrobiopterin dehydratase. (**C**) Functional enrichment analysis of differentially represented proteins in F versus C fed rats. GO terms for biological process (upper panel), molecular function (middle panel) and cellular component (lower panel) are reported.

Differentially represented proteins in groups F and C were analyzed with the STRING tool, using as a template the UniProtKB database of *R. norvegicus*; this allowed for predicting a functional protein association map, which was characterized by two ramified networks including 19 nodes and linking together 11 components, plus eight non-associated species (Figure 2B). On the other hand, functional analysis of differentially represented components according to KEGG metabolic pathway classification highlighted selective enrichment of proteins mainly involved in cholesterol metabolism, ferroptosis, oxidative phosphorylation, Parkinson's disease, and necroptosis. Several deregulated proteins belong to the GO Cellular Component Mitochondrial membrane, Mitochondrial respiratory chain complex I, and Mitochondrial outer membrane (Figure 2C).

3.2. Reduced Amount of Mitochondrial Respiratory Complexes, PGC-1α and VDAC-1 and Higher Level of PINK-1 in Hypothalamus of Fructose-Fed Adolescent Rats

Proteomic investigation revealed that fructose feeding determined a down-representation of specific proteins belonging to mitochondrial respiratory complexes I (Ndufs1, Ndufb9, and Ndufb10), IV (Cox6b1), and V (Atp5mj) (Figure 2A). With the aim to validate these diet-induced alterations, we further assessed by western blotting the abundance of complexes I–V. In line with proteomic data, the amount of all respiratory complexes was lower in fructose-fed than in control rats ($p < 0.01$; Figure 3), strongly suggesting that a short-term fructose diet generates early quantitative alterations of the oxidative phosphorylation system.

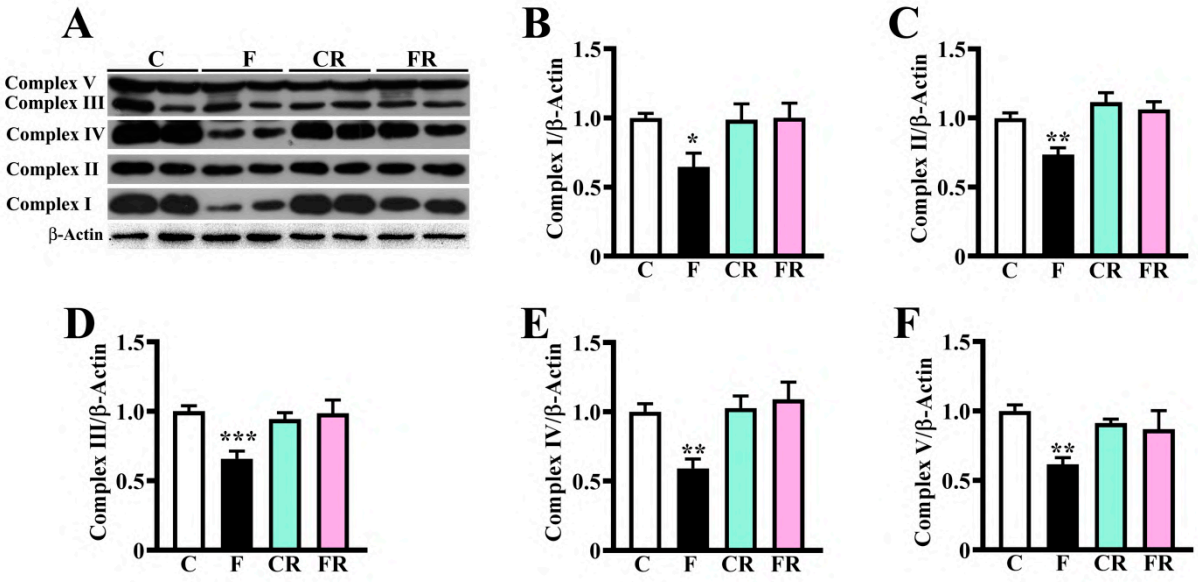

Figure 3. Mitochondrial respiratory complexes amount in rat hypothalamus. The amount of mitochondrial complex I, II, III, IV, and V was measured in protein extracts from the hypothalamus of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Samples were analyzed by 12.5% SDS-PAGE and western blotting. After the detection of immunocomplexes (by mouse anti-OXPHOS and GAM-HRP IgGs) the membrane was stripped and treated with anti-β-actin IgG as the loading control. (**A**) Representative western blotting of respiratory complexes. Panels B-E show results from densitometric analysis. (**B**) Complex I amount relative β-actin; (**C**) Complex II amount relative to β-actin; (**D**) Complex III amount relative to β-actin; (**E**) Complex IV amount relative to β-actin; (**F**) Complex IV amount relative to β-actin; (**F**): Complex V amount relative to β-actin. Data shown are reported as means ± SEM of eight animals from each group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus C (one-way Anova followed by Bonferroni post-test).

Based on these evidences, we measured the protein levels of PGC-1α, which is a critical coregulator of transcription factors participating in the regulation of mitochondrial biogenesis and respiration [42–44], cellular metabolism [45], and detoxification of reactive

oxygen species (ROS) produced by mitochondrial respiration [46,47], thus exerting a global positive impact on oxidative metabolism. The amount of PGC-1α was lower in F than the control group ($p < 0.001$; Figure 4A), in line with results on corresponding respiratory complexes. Since the decreased abundance of respiratory complexes and PGC-1α suggested a whole damage of the mitochondrial compartment, we further investigated whether fructose feeding affects the levels of PINK-1, which is a protein involved in the modulation of mitophagy [48]. A significant increase of this protein was evidenced in the F group ($p < 0.01$; Figure 4B), strongly supporting the hypothesis that the fructose diet was also associated with mitophagy activation and decreased mitochondrial biogenesis.

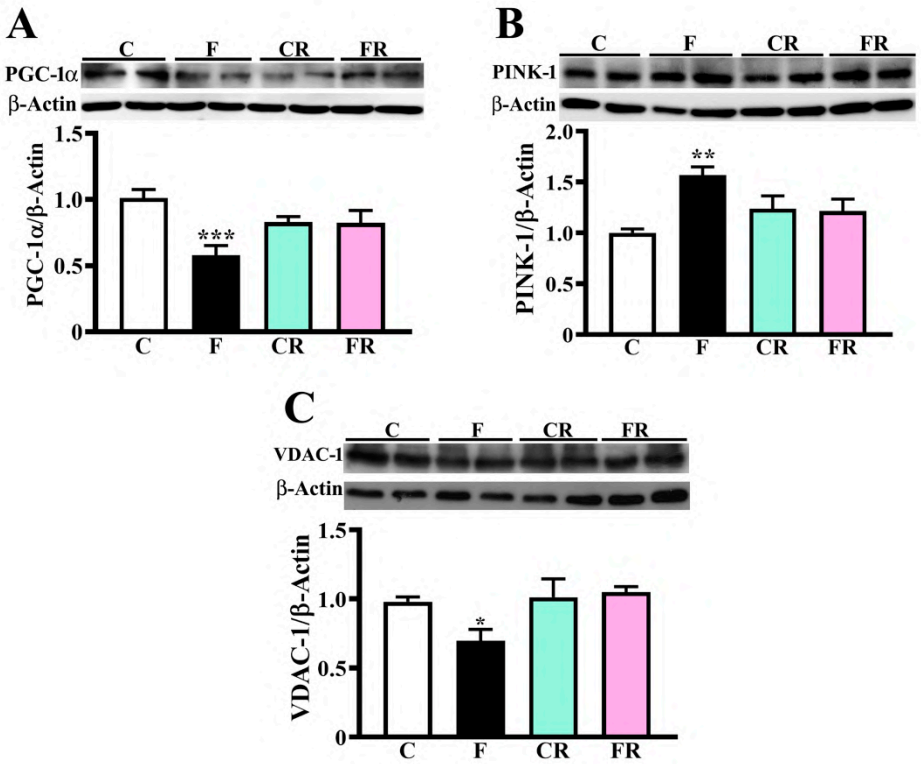

Figure 4. PGC-1α, PINK-1 and VDAC-1 levels in rat hypothalamus. PGC-1α (**A**), PINK-1 (**B**), and VDAC-1 (**C**) were quantified in protein extracts from the hypothalamus of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Samples were analyzed by 10% (PGC-1α) or 12.5% (PINK-1, VDAC-1) SDS-PAGE and western blotting. After detection of immunocomplexes [by rabbit anti-PGC-1α and GAR-HRP IgGs (**A**), or mouse anti-PINK-1 and GAM-HRP IgGs (**B**), or mouse anti-VDAC-1 and GAM-HRP IgGs (**C**)], the membranes were stripped and treated with anti-β-actin as loading control. Data shown are means ± SEM of eight animals from each group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus C (one-way Anova followed by Bonferroni post-test).

Proteomic results also revealed a significant decrease in the abundance of three voltage-dependent anion channel (VDAC) proteins (Figure 2A), namely VDAC-1, 2 and 3, which are placed in the outer mitochondrial membrane, and whose alteration is known to contribute to pathological states [49]. As shown in Figure 4C, VDAC-1 levels were reduced in fructose-fed rats ($p < 0.05$), confirming proteomic data, and also suggesting the occurrence of an impairment in the communication between the mitochondrial matrix and the cytosol, and a possible alteration of the mitochondrial functions.

The switch to the control diet determined the rescue of all the above-described protein changes, as demonstrated by the finding of no difference in the levels of these components was observed between CR and FR rats (Figures 2B, 3 and 4).

3.3. Increased Levels of Inflammatory and Oxidative Stress Markers in Hypothalamus of Fructose-Fed Adolescent Rats

Proteomic results also revealed a significant fructose-associated increase of S-100B protein (Figure 2A), which is known to participate in the induction of the inflammatory cascade, astrocyte activation, and oxidative stress [50–52]. Therefore, we evaluated NF-kB activation and the levels of TNF-alpha, IL-6, and GFAP, to verify whether the increase of S-100B in fructose-fed rats is associated with a rise in hypothalamic inflammation. The degree of NFkB phosphorylation was measured as the ratio between phosphorylated and total NFkB (p-NFkB/NFkB), and then used as a marker of the activation of the NFkB pro-inflammatory signaling pathway. As shown in Figure 5A, the p-NFkB/NFkB ratio was found higher in fructose-fed than in control rats ($p < 0.0001$). Further, both TNF-alpha and IL-6 concentrations were higher in F than in the C group ($p < 0.01$; Figure 5B,C). Similarly, higher amounts of GFAP ($p < 0.001$; Figure 5D) and Hpt ($p < 0.0001$; Figure 5E), an inflammatory marker sensitive to nutritional status [37,38], were detected in the hypothalamus of F rats. Altogether, these results strongly suggested that the increase of S-100B levels observed as a result of short-term high-fructose feeding might be responsible for NF-kB activation in the hypothalamus, which in turn promotes inflammatory cytokine production and astrocyte activation, leading to a general condition of inflammation therein.

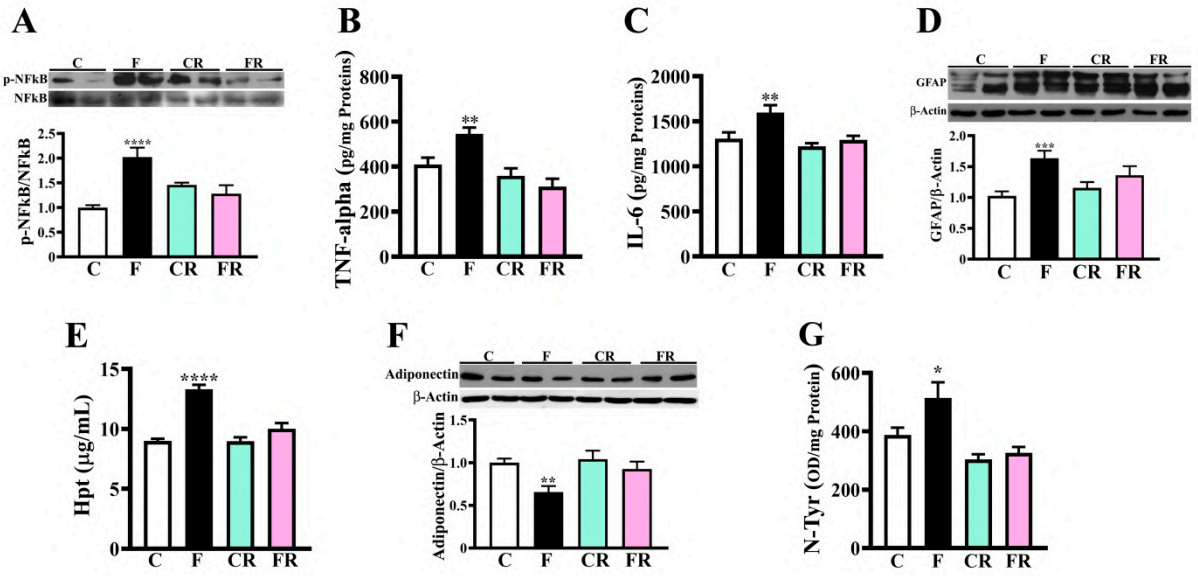

Figure 5. Evaluation of inflammatory and oxidative status in rat hypothalamus. (**A**) p-NFkB/NFkB ratio (representative western blotting and densitometric analysis). Samples were analyzed by 10% SDS-PAGE and western blotting. After rivelation of the immunocomplexes (by mouse anti-phosphoNFkB and GAM-HRP IgGs), the membrane was stripped and treated with mouse anti-NFkB and GAM-HRP IgGs. The amount of phosphorylated NFkB was expressed relative to the total NFkB level. Data shown are means ± SEM of eight animals from each group. (**B**) TNF-alpha and (**C**) IL-6 amount was titrated by sandwich ELISA on samples diluted 1:25 and following the manufacturer's instructions. Data are expressed as pg per mg of total proteins and reported as means ± SEM of eight different rats from each experimental group. (**D**) GFAP amount (representative western blotting and densitometric analysis). Samples were analyzed by 12.5% SDS-PAGE and western blotting. After the rivelation of immunocomplexes (by rabbit anti-GFAP and GAR-HRP IgGs), the membrane was stripped and re-incubated with anti-β-actin. Data shown are means ± SEM of eight animals from each group. (**E**) Hpt level was titrated by ELISA in samples diluted 1: 2000, 1:8000 and 1:25,000. Immunodetection was carried out with rabbit anti-Hpt and GAR-HRP IgGs. Data are means ± SEM of eight animals from each group. (**F**) Adiponectin amount (representative western blotting and densitometric analysis). Samples were analyzed by 12.5% SDS-PAGE and western blotting. After rivelation of the immunocomplexes (by rabbit anti-adiponectin and GAR-HRP IgGs), the membrane was stripped and

re-incubated with anti-β-actin. Data shown are means ± SEM of eight animals from each group. (**G**) N-Tyr amount was titrated by ELISA on samples diluted 1:1500, 1:3000 and 1:6000. Immunodetection was carried out with rabbit anti-N-Tyr and GAR-HRP IgGs. Data are expressed as OD per mg of total proteins and reported as means ± SEM of eight different rats from each experimental group. Control (C), fructose-fed (F), control rescued (CR), and fructose-rescued (FR) rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$ versus C (one-way Anova followed by Bonferroni post-test).

Finally, the concentration of adiponectin, which is an adipokine exerting antioxidant, anti-inflammatory, and neuroprotective effect [53,54], was lower in sugar-fed than in control rats ($p < 0.01$; Figure 5F). Similarly, plasma levels of adiponectin were found to decrease in F rats with respect to C ones ($p < 0.01$; Supplementary Materials Figure S3), with persistent lower levels in FR compared to the CR group ($p < 0.05$; Supplementary Materials Figure S3). In line with the observed reduction in F rats of adiponectin, fructose feeding was also associated with the increased concentration of N-Tyr ($p < 0.05$; Figure 5G), which is a marker of protein oxidation induced by peroxynitrite [55].

The switch to the control diet determined the rescue of all the above-reported protein changes, as demonstrated by the finding of no difference in the levels of these components between CR and FR rats (Figures 2B and 5).

3.4. Decreased Amount of Neuronal Intermediate Filaments, BDNF, and Synaptic Markers in Hypothalamus of Fructose-Fed Adolescent Rats

Proteomic analysis (Figure 2A) also showed a significant reduction of the abundance of neurofilament light protein, neurofilament medium protein, and alpha-internexin, which are subunits of neuronal intermediate filaments, namely neurofilaments, in fructose-fed rats compared to control rats. The amount of α-internexin was also measured by western blotting and this approach confirmed a significant decrease in protein levels in fructose-fed rats ($p < 0.01$; Figure 6A), with respect to control, validating proteomic data. As neurofilament proteins and α-internexin are the major components of neurons' cytoskeleton and their lack has already been related to neuronal loss [56], we may hypothesize that short-term fructose feeding might cause damage to nerve cells.

BDNF signaling participates in the modulation of different neurophysiological processes [57], such as synaptic function, dendritic spine maturation, and stabilization. Accordingly, we investigated whether the amounts of this neurotrophin in hypothalamic tissues are affected by fructose feeding. We observed that BDNF levels were halved in sugar-fed rats ($p < 0.01$; Figure 6B). Further, the amount of the full-length TrkB isoform, which is the high-affinity receptor of BDNF, was significantly lower in the F than in the C group ($p < 0.05$; Figure 6C), suggesting that short-term sugar feeding negatively impacts both BDNF production and signaling. These results are also in good agreement with proteomic data reporting a lower abundance of Sorcs2 protein in the F group compared with C one (Figure 2A). As Sorcs2 traffics TrkB to the postsynaptic membrane and interacts with TrkB, forming a complex that is essential for BDNF signalling [58], we may hypothesize that the observed fructose-associated decrease of SorCS2 should contribute to the alteration of neurotrophin functions.

Then, we evaluated the amount of presynaptic proteins synaptophysin and synaptotagmin, as well as postsynaptic protein PSD-95. High-fructose diet was associated with a significant reduction of both synaptophysin and synaptotagmin ($p < 0.01$ r; Figure 6D,E), as well as of PSD-95 ($p < 0.01$; Figure 5F).

The switch to the control diet fully rescued all above-reported protein alterations, as demonstrated by the finding of no difference in the levels of these components was detected between CR and FR rats (Figures 2B and 6).

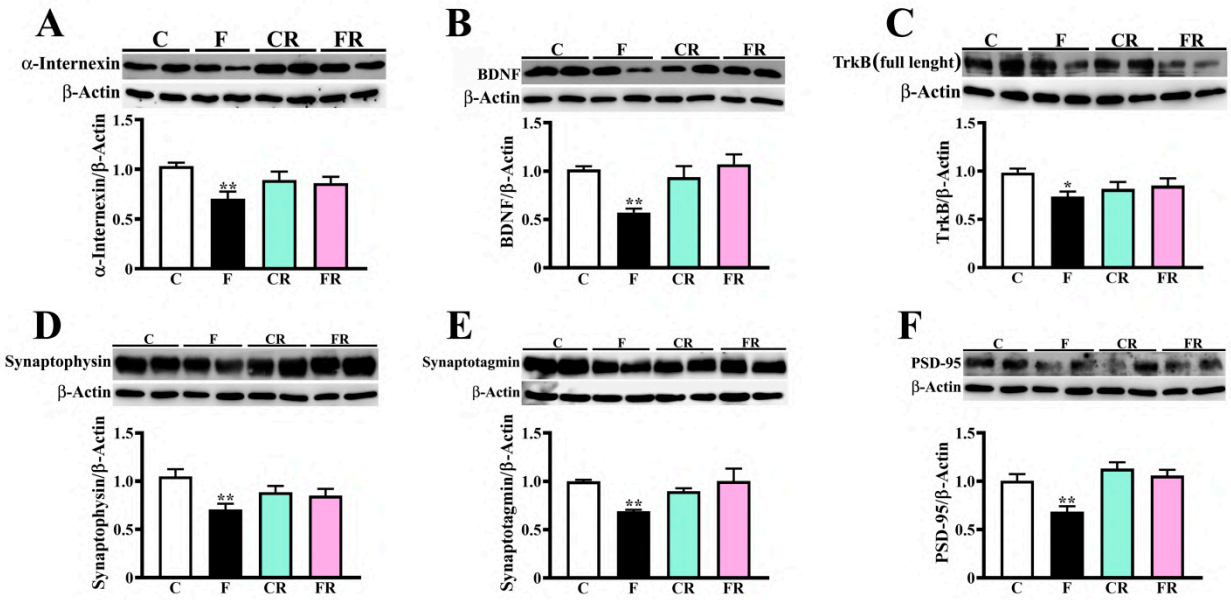

Figure 6. Evaluation of α-internexin, BDNF, TrkB, and synaptic proteins in rat hypothalamus. (**A**) α-internexin, (**B**) BDNF, (**C**) full-length TrkB isoform, (**D**) synaptophysin, (**E**) synaptotagmin, and (**F**) PSD-95 were measured in protein extracts from the hypothalamus of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), young-adult fructose-rescued (FR) rats. Samples were analyzed by 10% (α-internexin, TrkB, synaptotagmin, PSD-95) or 12.5% (BDNF, synaptophysin) SDS-PAGE and western blotting. Immunocomplexes were detected by rabbit mouse anti-α-internexin and GAM-HRP IgGs (**A**), or rabbit anti-BDNF (**B**), rabbit anti-TrkB (**C**), rabbit anti-synaptophysin (**D**), rabbit anti-synaptotagmin (**E**), rabbit anti-PSD-95 (**F**) and GAR-HRP IgGs. After detection, the membranes were stripped and treated with anti- β-actin. Means ± SEM of eight animals from each experimental group are shown. * $p < 0.05$, ** $p < 0.01$ versus C (one-way Anova followed by Bonferroni post-test).

4. Discussion

High-fructose intake has been reported to have numerous unhealthy consequences within the spectrum of metabolic syndromes, impacting genes related to several metabolic pathologies, and correlating with increasing incidence of neurologic diseases [5,59–61]. Several studies on rodent models have shown that fructose and glucose modulate in a different manner the cerebral pathways regulating appetite and feeding behavior [62]. As a matter of fact, central administration of fructose stimulates feeding, while glucose induces satiety in rodents [63,64]; it was also proposed that fructose-containing diets, as provoking smaller raises of insulin and leptin secretion compared to glucose [65,66], exert a lower inhibition on orexigenic neurons [67]. Long-term fructose feeding was shown to be associated with a high preference and motivation for the sugar-based diet [68], to elicit leptin resistance in rats, due to a defective transport of the protein through the blood-brain barrier [69,70], and also to determine upregulated expression of neuropeptide Y (NPY) [70]. On the other hand, a short-term fructose diet stimulates the production of ghrelin, and concomitantly down-regulates the satiety signal peptide YY3-36 and the orexigenic NPY, determining a cooperative effect in the modulation of appetite and food intake [71].

Since the hypothalamus is the master center for control of brain and body homeostasis, it was recently proposed that the effects of fructose on hypothalamic metabolism might play a key role in triggering metabolic disorders, which in turn may determine neurological effects, ultimately affecting brain functions and behavior [5,72]. However, current knowledge mostly refers to prolonged dietary treatments, whereas no information is available about the effects of short-term fructose feeding. Our challenge was to identify molecular mechanisms early influenced by sugar feeding in the hypothalamus through the

investigation of corresponding protein changes associated with a short-term fructose-rich diet, focusing on a critical phase of growth, namely adolescence.

The proteomic analysis allowed us to portray early quantitative molecular changes associated with fructose consumption, evidencing 19 proteins differentially represented between control and treated rats. A strong alteration was observed in the amount of specific mitochondrial proteins, such as the components of respiratory complexes I (Ndufs1, Ndufb9, and Ndufb10), IV (Cox6b1), and V (ATP5MJ), whose levels were reduced by about 30%. These proteins are part of complexes placed in the inner mitochondrial membrane and are responsible for oxidative phosphorylation as well as of ATP production [73]. The western blotting analysis demonstrated a significant reduction in the level of protein components of complex V (ATP5A), IV (MTCO1), and I (NDUFB8), validating proteomic results, and also showed a significant decrease of specific elements of complexes II and III. Considering that complex I protein NDUFB8 and complex IV protein MTCO1 are widely used as biomarkers of mitochondrial content [74,75], the finding of a quantitative reduction of components of all mitochondrial respiratory complexes was suggestive of a deleterious effect of a short-term high-fructose diet on mitochondrial biogenesis. Our hypothesis of a fructose-associated alteration of mitochondrial abundance was supported by the finding of the decreased abundance of the biogenesis-related protein PGC-1α, and higher amounts of the mitophagy marker PINK-1 [48,76] in the hypothalamus of treated rats. In this context, although proteomic investigation did not reveal changes in the abundance of proteins belonging to the mitochondrial fission and fusion machinery (data not shown), future studies on mitochondrial proteome and respiration might contribute to better clarifying fructose effect on mitochondria dynamics and quality control mechanisms, which are essential for maintaining their proper environment and functioning.

The proteomic analysis also evidenced a strong decrease in the abundance of VDAC-1, VDAC-2, and VDAC-3 in short-term high-fructose-fed rats, which was confirmed in the case of VDAC-1 by immunoblotting. VDAC proteins are mitochondrial porins localized on the outer membrane, involved in the exchange of molecules between the mitochondrial matrix and cytoplasm, as well as in the docking of cytosolic and mitochondrial proteins [49]. In particular, VDAC-1 is considered a gatekeeper for mitochondrial functions, and its alteration was reported to contribute to the pathogenesis of several diseases [49]. Since VDAC-1 acts as a hub protein modulating the integration between mitochondrial and other cellular activities [77], our results on the lower abundance of VDAC-1 in fructose-fed rats led us to hypothesize that a sugar-rich diet negatively impacts the whole mitochondrial compartment and, likely, on hypothalamic cells functions. The observed compromising of the mitochondrial compartment following the fructose feeding represents a deleterious culprit for hypothalamic functions but also an alarm of the possible predisposition to the development of degenerative phenomena in the long-term, as mitochondrial dysfunction and impaired organelle dynamics are linked to neurodegenerative and metabolic diseases [78–80]. We previously reported a fructose-induced impairment of complex II activity in the hippocampus of adolescent rats [13]. Therefore, an interesting goal of future research will be investigating whether short-term fructose feeding alters hypothalamic mitochondrial respiratory functions by evaluating possible dysfunction in electron transport, ADP phosphorylation, and leak respiration across the inner mitochondrial membrane.

Interestingly, proteomic experiments also highlighted increased levels of S-100B protein in high-fructose-fed rats, thus hinting at an inflammatory status in these animals. Indeed, the S-100B protein, when overproduced by activated glia, acts as a pro-inflammatory cytokine, and contributes to neuroinflammation heightening and neuronal dysfunction [50]. Hence, this protein promotes the migration and activation of microglia [81], also inducing TNF-alpha expression therein [52,82] and induces an autocrine loop in astrocytes, which results in the stimulation of IL-6 and TNF-alpha secretion [51]. In agreement with the detected increase of S-100B protein in the hypothalamus of high-fructose-fed rats, we observed an enhanced NF-kB activation, higher levels of the astrocytic marker GFAP, and the rise of two inflammatory cytokines, TNF-α and IL-6, as well as of Hpt. These results

led us to hypothesize that the fructose-associated gain of S-100B is a trigger event driving hypothalamic inflammation. It is worth mentioning that hypothalamic inflammation is considered a potential trigger for the deregulation of mechanisms involved in food control and energy metabolism [28,83,84]. As a matter of fact, a long-term fructose diet was reported to induce increased IL-6 and TNF-alpha expression in mice hypothalamus, together with down-regulation of the expression of both the anorexigenic proopio-melanocortin (POMC) and the orexigenic NPY [85]. As above mentioned, several animal studies demonstrated the orexigenic effect of fructose [28]. Indeed, long-term fructose feeding in rats was associated with increased expression of the NPY gene [26,70,86], reduced POMC expression and impairment in the melanocortin system [26], and augmented gene expression of the agouti-related peptide [70,86]. However, it was pointed out that results regarding fructose effects on food intake and energy metabolism modulation are often contrasting and essentially depend on the animal model, intake doses, mode, and duration of administration [62]. In our experimental model, dietary treatment was not associated with changes in body weight and daily energy intake [87]. Accordingly, the proteomic investigation did not reveal POMC abundance changes (data not shown), maybe because of the young age of rats or the short duration of diet administration. Nevertheless, we cannot exclude that hypothalamic inflammation might be linked to alterations in the expression of orexigenic and/or anorexigenic genes; future analyses will be critical to clarify this issue.

The hypothesis of a diet-associated hypothalamic impairment was also supported by the finding, in fructose-fed rats, of decreased levels of adiponectin, a protein playing physiological functions in the brain, regulating synaptic plasticity [88], preventing oxidative stress and suppressing inflammatory cascade [89–91]. Hence, the decrease of adiponectin in high-fructose-fed rats might represent a further molecular contribution to hypothalamic inflammation, also depriving this region of protection against the pro-oxidant effect of inflammatory cytokines. Although recent studies demonstrated adiponectin expression in the hypothalamus of mice, beavers, or female pigs [92–95], we cannot exclude that adipocytes are the source of the protein we measured in the hypothalamus. Indeed, peripheral adiponectin can cross the blood-brain barrier reaching the brain [96–98]. Accordingly, we measured lower adiponectin levels in the plasma of sugar-fed rats. Further, as PGC-1α is also known to participate in the detoxification of ROS [46,47], the observed decrease of both this protein and adiponectin in fructose-fed rats was also suggestive of a condition of oxidative stress in the hypothalamus of these animals. Indeed, lower levels of N-Tyr corroborated the alteration of the redox balance in high-fructose-fed rats.

Proteomic results also revealed a decrease in the amount of three protein components (neurofilament light, NF-L; neurofilament medium, NF-M; alpha-internexin) of neurofilaments (NFs) in fructose-fed rats. In the case of alpha-internexin, these results were confirmed by western blotting. NFs play a key structural role in neurons [99,100], are essential for synaptic functions [101,102], and form cellular scaffolds involved in the docking and organization of synaptic vesicles, endosomes, and endoplasmic reticulum [103]. Because of the multiple key functions of NFs in neurons, the observed alteration of the cytoskeleton scaffold led us to suppose that the damaging effect of a high-fructose diet might also lead to a disruption of the neuronal network, with possible deleterious impact on corresponding functions. In addition to the above-mentioned results on NF-L, our proteomic analysis notably demonstrated a slight (about 18%) but significant decrease in fructose-fed rats of CaMKII2b, which is a neuronal isoform of CAMKII crucial for both growth and arborization of dendrites during the developmental phase [104]. This protein also promotes synapse and spine formation and elongation [105], controls dendritic morphology, neurite extension, and synapse number, and is essential for long-term plasticity, learning, and memory consolidation [106]. This finding further supports the hypothesis of a fructose-induced neuronal dysfunction, which could also affect synaptic functions.

In fructose-fed rats, we also observed a decreased amount of both BDNF and its high-affinity receptor TrkB, in line with data previously obtained in the prefrontal cortex of adult [14] and young [107] rats fed the same sugar-rich diet. In this context, proteomics also

detected a concomitant decrease of SorCS2, which is a protein localized within synaptic vesicles and at the post-synaptic density of dendrites [58,108]. SorCS2 traffics TrkB to the postsynaptic membrane, and it was reported to interact with TrkB, forming a complex that is fundamental for several BDNF-dependent processes such as hippocampal long-term potentiation and changes in dendritic complexity and spine density [58]. As BDNF-TrkB signaling is crucial in supporting neuronal survival, differentiation, and growth, as well as synaptic transmission [57], the decrease of both neurotrophin and receptor corroborates the idea of a fructose-induced disruption of synaptic plasticity. In line with this hypothesis, we observed lower levels of synaptophysin, synaptotagmin, and post-synaptic density protein 95 (PSD-95) in the hypothalamus of F rats, compared to control ones. Since the TrkB receptor is associated with PSD-95, and BDNF/TrkB signaling increases both the recruitment of PSD-95 to synapses [109] and its localization at dendritic spines [110], the observed decrease of both BDNF and TrkB in F rats may be responsible for the decrement of PSD-95, confirming a fructose-associated impairment of BDNF-depending molecular mechanisms. The decreased amount of these synaptic markers in fructose-fed rats, together with the concomitant reduction of levels of BDNF, neurofilament proteins, and PGC-1α, which is known to participate in the regulation of brain plasticity by modulating spinogenesis and synaptogenesis [111], prompted us to suggest that short-term sugar feeding might negatively impact on synapse number, and may cause synaptic dysfunctions and impaired brain network activities.

5. Conclusions

To the best of our knowledge, this is the first study examining both the effect of a short-term fructose-rich diet on the hypothalamus, throughout adolescent development, and the possible persistence of sugar-induced changes until young adulthood, after returning to a balanced diet. Overall, the results here reported indicating that a diet rich in fructose, even if accomplished for short period, highly affects mitochondrial and cytoskeletal compartments, as well as the level of specific markers of brain function and plasticity. Interestingly, unlike what we observed in the frontal cortex of fructose-fed rats [107], the fructose-driven changes detected in the hypothalamus were fully rescued after switching to the control diet. Further studies comparing the effect of fructose and other sugars will be important to evaluate the possible use of alternative sugars for processed food.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15020475/s1. Table S1: Identification details of differentially represented proteins; Figure S1: Proteomic analysis of hypothalamic tissues; Figure S2: Heat-map hierarchical cluster analysis; Figure S3: Adiponectin level in plasma.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD038834. All data supporting the findings of this study are available within the article and its supplementary information files.

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References

1. Gómez-Pinilla, F. Brain foods: The effects of nutrients on brain function. *Nat. Rev. Neurosci.* **2008**, *9*, 568–578. [CrossRef] [PubMed]
2. Carneiro, L.; Pellerin, L. Nutritional Impact on Metabolic Homeostasis and Brain Health. *Front. Neurosci.* **2022**, *15*, 767405. [CrossRef] [PubMed]
3. Zhang, Q.; Jin, K.; Chen, B.; Liu, R.; Cheng, S.; Zhang, Y.; Lu, J. Overnutrition Induced Cognitive Impairment: Insulin Resistance, Gut-Brain Axis, and Neuroinflammation. *Front. Neurosci.* **2022**, *16*, 884579. [CrossRef] [PubMed]
4. Johnson, R.J.; Gomez-Pinilla, F.; Nagel, M.; Nakagawa, T.; Rodriguez-Iturbe, B.; Sanchez-Lozada, L.G.; Tolan, D.R.; Lanaspa, M.A. Cerebral Fructose Metabolism as a Potential Mechanism Driving Alzheimer's Disease. *Front Aging Neurosci.* **2020**, *12*, 560865. [CrossRef] [PubMed]
5. Gomez-Pinilla, F.; Cipolat, R.P.; Royes, L.F.F. Dietary fructose as a model to explore the influence of peripheral metabolism on brain function and plasticity. *Biochim. Biophys. Acta Mol. Basis Dis.* **2021**, *1867*, 166036. [CrossRef]
6. Vos, M.B.; Kimmons, J.E.; Gillespie, C.; Welsh, J.; Blanck, H.M. Dietary fructose consumption among US children and adults: The third national health and nutrition examination survey. *Medscape J. Med.* **2008**, *10*, 160.
7. Jensen, T.; Abdelmalek, M.F.; Sullivan, S.; Nadeau, K.J.; Green, M.; Roncal, C.; Nakagawa, T.; Kuwabara, M.; Sato, Y.; Kang, D.H.; et al. Fructose and sugar: A major mediator of non-alcoholic fatty liver disease. *J. Hepatol.* **2018**, *68*, 1063–1075. [CrossRef]
8. Mirtschink, P.; Jang, C.; Arany, Z.; Krek, W. Fructose metabolism, cardiometabolic risk, and the epidemic of coronary artery disease. *Eur. Heart J.* **2018**, *39*, 2497–2505. [CrossRef]
9. Taskinen, M.R.; Packard, C.J.; Borén, J. Dietary fructose and the metabolic syndrome. *Nutrients* **2019**, *11*, 1987. [CrossRef]
10. Cheng, H.; Zhou, J.; Sun, Y.; Zhan, Q.; Zhang, D. High fructose diet: A risk factor for immune system dysregulation. *Hum. Immunol.* **2022**, *83*, 538–546. [CrossRef]
11. Spagnuolo, M.S.; Iossa, S.; Cigliano, L. Sweet but Bitter: Focus on Fructose Impact on Brain Function in Rodent Models. *Nutrients* **2020**, *13*, 1. [CrossRef]
12. Cigliano, L.; Spagnuolo, M.S.; Crescenzo, R.; Cancelliere, R.; Iannotta, L.; Mazzoli, A.; Liverini, G.; Iossa, S. Short-Term Fructose Feeding Induces Inflammation and Oxidative Stress in the Hippocampus of Young and Adult Rats. *Mol. Neurobiol.* **2018**, *55*, 2869–2883. [CrossRef]
13. Mazzoli, A.; Spagnuolo, M.S.; Nazzaro, M.; Gatto, C.; Iossa, S.; Cigliano, L. Fructose Removal from the Diet Reverses Inflammation, Mitochondrial Dysfunction, and Oxidative Stress in Hippocampus. *Antioxidants* **2021**, *10*, 487. [CrossRef] [PubMed]
14. Spagnuolo, M.S.; Bergamo, P.; Crescenzo, R.; Iannotta, L.; Treppiccione, L.; Iossa, S.; Cigliano, L. Brain Nrf2 pathway, autophagy, and synaptic function proteins are modulated by a short-term fructose feeding in young and adult rats. *Nutr. Neurosci.* **2020**, *23*, 309–320. [CrossRef]
15. Van der Borght, K.; Köhnke, R.; Göransson, N.; Deierborg, T.; Brundin, P.; Erlanson-Albertsson, C.; Lindqvist, A. Reduced Neurogenesis in the Rat Hippocampus Following High Fructose Consumption. *Regul. Pept.* **2011**, *167*, 26–30. [CrossRef] [PubMed]
16. Hsu, T.M.; Konanur, V.R.; Taing, L.; Usui, R.; Kayser, B.D.; Goran, M.I.; Kanoski, S.E. Effects of sucrose and high fructose corn syrup consumption on spatial memory function and hippocampal neuroinflammation in adolescent rats. *Hippocampus* **2015**, *25*, 227–239. [CrossRef] [PubMed]
17. Wu, H.W.; Ren, L.F.; Zhou, X.; Han, D.W. A high-fructose diet induces hippocampal insulin resistance and exacerbates memory deficits in male Sprague-Dawley rats. *Nutr. Neurosci.* **2015**, *18*, 323–328. [CrossRef]
18. Harrell, C.S.; Zainaldin, C.; McFarlane, D.; Hyer, M.M.; Stein, D.; Sayeed, I.; Neigh, G.N. High-fructose diet during adolescent development increases neuroinflammation and depressive-like behavior without exacerbating outcomes after stroke. *Brain Behav. Immun.* **2018**, *73*, 340–351. [CrossRef]

19. Clark, K.A.; Alves, J.M.; Jones, S.; Yunker, A.G.; Luo, S.; Cabeen, R.P.; Angelo, B.; Xiang, A.H.; Page, K.A. Dietary Fructose Intake and Hippocampal Structure and Connectivity during Childhood. *Nutrients* **2020**, *12*, 909. [CrossRef]
20. Elmquist, J.K.; Coppari, R.; Balthasar, N.; Ichinose, M.; Lowell, B.B. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *J. Comp. Neurol.* **2005**, *493*, 63–71. [CrossRef]
21. Marty, N.; Dallaporta, M.; Thorens, B. Brain glucose sensing, counterregulation, and energy homeostasis. *Physiology* **2007**, *22*, 241–251. [CrossRef] [PubMed]
22. Harrell, C.S.; Burgado, J.; Kelly, S.D.; Johnson, Z.P.; Neigh, G.N. High-fructose diet during periadolescent development increases depressive-like behavior and remodels the hypothalamic transcriptome in male rats. *Psychoneuroendocrinology* **2015**, *62*, 252–264. [CrossRef] [PubMed]
23. Zhang, Q.Y.; Pan, Y.; Wang, R.; Kang, L.L.; Xue, Q.C.; Wang, X.N.; Kong, L.D. Quercetin inhibits AMPK/TXNIP activation and reduces inflammatory lesions to improve insulin signaling defect in the hypothalamus of high fructose-fed rats. *J. Nutr. Biochem.* **2014**, *25*, 420–428. [CrossRef] [PubMed]
24. Kovačević, S.; Nestorov, J.; Matić, G.; Elaković, I. Chronic Stress Combined with a Fructose Diet Reduces Hypothalamic Insulin Signaling and Antioxidative Defense in Female Rats. *Neuroendocrinology* **2019**, *108*, 278–290. [CrossRef]
25. Bermejo-Millo, J.C.; Guimarães, M.R.M.; de Luxán-Delgado, B.; Potes, Y.; Pérez-Martínez, Z.; Díaz-Luis, A.; Caballero, B.; Solano, J.J.; Vega-Naredo, I.; Coto-Montes, A. High-Fructose Consumption Impairs the Redox System and Protein Quality Control in the Brain of Syrian Hamsters: Therapeutic Effects of Melatonin. *Mol. Neurobiol.* **2018**, *55*, 7973–7986. [CrossRef]
26. Li, J.M.; Ge, C.X.; Xu, M.X.; Wang, W.; Yu, R.; Fan, C.Y.; Kong, L.D. Betaine recovers hypothalamic neural injury by inhibiting astrogliosis and inflammation in fructose-fed rats. *Mol. Nutr. Food Res.* **2015**, *59*, 189–202. [CrossRef]
27. Lu, W.; Xu, Y.; Shao, X.; Gao, F.; Li, Y.; Hu, J.; Zuo, Z.; Shao, X.; Zhou, L.; Zhao, Y.; et al. Uric acid produces an inflammatory response through activation of NF-κB in the hypothalamus: Implications for the pathogenesis of metabolic disorders. *Sci. Rep.* **2015**, *5*, 12144. [CrossRef]
28. Cargnin-Carvalho, A.; de Mello, A.H.; Bressan, J.B.; Backes, K.M.; Uberti, M.F.; Fogaça, J.B.; da Rosa Turatti, C.; Cavalheiro, E.K.F.F.; Vilela, T.C.; Rezin, G.T. Can fructose influence the development of obesity mediated through hypothalamic alterations? *J. Neurosci. Res.* **2020**, *98*, 1662–1668. [CrossRef]
29. Bauer, L.L.; Murphy, M.R.; Wolf, B.W.; Fahey, G.C., Jr. Estimates of starch digestion in the rat small intestine differ from those obtained using in vitro time-sensitive starch fractionation assays. *J. Nutr.* **2003**, *133*, 2256–2261. [CrossRef]
30. Sasaki, T.; Sotome, I.; Okadome, H. In vitro starch digestibility and in vivo glucose response of gelatinized potato starch in the presence of non-starch polysaccharides. *Starch* **2015**, *67*, 415–423. [CrossRef]
31. Spagnuolo, M.S.; Maresca, B.; Mollica, M.P.; Cavaliere, G.; Cefaliello, C.; Trinchese, G.; Esposito, M.G.; Scudiero, R.; Crispino, M.; Abrescia, P.; et al. Haptoglobin increases with age in rat hippocampus and modulates Apolipoprotein E mediated cholesterol trafficking in neuroblastoma cell lines. *Front. Cell Neurosci.* **2014**, *8*, 212. [CrossRef] [PubMed]
32. Visconti, S.; D'Ambrosio, C.; Fiorillo, A.; Arena, S.; Muzi, C.; Zottini, M.; Aducci, P.; Marra, M.; Scaloni, A.; Camoni, L. Overexpression of 14-3-3 proteins enhances cold tolerance and increases levels of stress-responsive proteins of Arabidopsis plants. *Plant Sci.* **2019**, *289*, 110215. [CrossRef] [PubMed]
33. Spagnuolo, M.S.; Pallottini, V.; Mazzoli, A.; Iannotta, L.; Tonini, C.; Morone, B.; Ståhlman, M.; Crescenzo, R.; Strazzullo, M.; Iossa, S.; et al. A Short-Term Western Diet Impairs Cholesterol Homeostasis and Key Players of Beta Amyloid Metabolism in Brain of Middle-Aged Rats. *Mol. Nutr. Food Res.* **2020**, *64*, e2000541. [CrossRef] [PubMed]
34. Spagnuolo, M.S.; Maresca, B.; La Marca, V.; Carrizzo, A.; Veronesi, C.; Cupidi, C.; Piccoli, T.; Maletta, R.G.; Bruni, A.C.; Abrescia, P.; et al. Haptoglobin interacts with apolipoprotein E and beta-amyloid and influences their crosstalk. *ACS Chem. Neurosci.* **2014**, *5*, 837–847. [CrossRef] [PubMed]
35. Crescenzo, R.; Spagnuolo, M.S.; Cancelliere, R.; Iannotta, L.; Mazzoli, A.; Gatto, C.; Iossa, S.; Cigliano, L. Effect of Initial Aging and High-Fat/High-Fructose Diet on Mitochondrial Bioenergetics and Oxidative Status in Rat Brain. *Mol. Neurobiol.* **2019**, *56*, 7651–7663. [CrossRef]
36. Spagnuolo, M.S.; Donizetti, A.; Iannotta, L.; Aliperti, V.; Cupidi, C.; Bruni, A.C.; Cigliano, L. Brain-derived neurotrophic factor modulates cholesterol homeostasis and Apolipoprotein E synthesis in human cell models of astrocytes and neurons. *J. Cell Physiol.* **2018**, *233*, 6925–6943. [CrossRef]
37. Spagnuolo, M.S.; Mollica, M.P.; Maresca, B.; Cavaliere, G.; Cefaliello, C.; Trinchese, G.; Scudiero, R.; Crispino, M.; Cigliano, L. High fat diet and inflammation—Modulation of haptoglobin level in rat brain. *Front. Cell Neurosci.* **2015**, *9*, 479. [CrossRef]
38. Mazzoli, A.; Spagnuolo, M.S.; Gatto, C.; Nazzaro, M.; Cancelliere, R.; Crescenzo, R.; Iossa, S.; Cigliano, L. Adipose Tissue and Brain Metabolic Responses to Western Diet-Is There a Similarity between the Two? *Int. J. Mol. Sci.* **2020**, *21*, 786. [CrossRef]
39. Aquilano, K.; Sciarretta, F.; Turchi, R.; Li, B.H.; Rosina, M.; Ceci, V.; Guidobaldi, G.; Arena, S.; D'Ambrosio, C.; Audano, M.; et al. Low-protein/high-carbohydrate diet induces AMPK-dependent canonical and non-canonical thermogenesis in subcutaneous adipose tissue. *Redox Biol.* **2020**, *36*, 101633. [CrossRef]
40. Li, Z.; Adams, R.M.; Chourey, K.; Hurst, G.B.; Hettich, R.L.; Pan, C. Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. *J. Proteome Res.* **2012**, *11*, 1582–1590. [CrossRef]
41. Megger, D.A.; Pott, L.L.; Ahrens, M.; Padden, J.; Bracht, T.; Kuhlmann, K.; Eisenacher, M.; Meyer, H.E.; Sitek, B. Comparison of label-free and label-based strategies for proteome analysis of hepatoma cell lines. *Biochim. Biophys. Acta* **2014**, *1844*, 967–976. [CrossRef] [PubMed]

42. Wu, Z.; Puigserver, P.; Andersson, U.; Zhang, C.; Adelmant, G.; Mootha, V.; Troy, A.; Cinti, S.; Lowell, B.; Scarpulla, R.C.; et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **1999**, *98*, 115–124. [CrossRef] [PubMed]
43. Scarpulla, R.C. Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. *Gene* **2002**, *286*, 81–89. [CrossRef] [PubMed]
44. Wareski, P.; Vaarmann, A.; Choubey, V.; Safiulina, D.; Liiv, J.; Kuum, M.; Kaasik, A. PGC-1α and PGC-1B Regulate Mitochondrial Density in Neurons. *J. Biol. Chem.* **2009**, *284*, 21379–21385. [CrossRef]
45. Martinez-Redondo, V.; Pettersson, A.T.; Ruas, J.L. The hitchhiker's guide to PGC-1alpha isoform structure and biological functions. *Diabetologia* **2015**, *58*, 1969–1977. [CrossRef]
46. Valle, I.; Alvarez-Barrientos, A.; Arza, E.; Lamas, S.; Monsalve, M. PGC-1alpha regulates the mitochondrial antioxidant defense system in vascular endothelial cells. *Cardiovasc. Res.* **2005**, *66*, 562–573. [CrossRef]
47. St-Pierre, J.; Drori, S.; Uldry, M.; Silvaggi, J.M.; Rhee, J.; Jäger, S.; Handschin, C.; Zheng, K.; Lin, J.; Yang, W.; et al. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* **2006**, *127*, 397–408. [CrossRef]
48. Pickrell, A.M.; Youle, R.J. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron* **2015**, *85*, 257–273. [CrossRef]
49. Camara, A.K.S.; Zhou, Y.; Wen, P.C.; Tajkhorshid, E.; Kwok, W.M. Mitochondrial VDAC1: A Key Gatekeeper as Potential Therapeutic Target. *Front. Physiol.* **2017**, *8*, 460. [CrossRef]
50. Van Eldik, L.J.; Wainwright, M.S. The Janus face of glial-derived S100B: Beneficial and detrimental functions in the brain. *Rest. Neurol. Neurosci.* **2003**, *21*, 97–108.
51. Ponath, G.; Schettler, C.; Kaestner, F.; Voigt, B.; Wentker, D.; Arolt, V.; Rothermundt, M. Autocrine S100B effects on astrocytes are mediated via RAGE. *J. Neuroimmunol.* **2007**, *184*, 214–222. [CrossRef] [PubMed]
52. Bianchi, R.; Giambanco, I.; Donato, R. S100B/RAGE-dependent activation of microglia via NF-kappaB and AP-1 Co-regulation of COX-2 expression by S100B, IL-1beta and TNF-alpha. *Neurobiol. Aging* **2010**, *31*, 665–677. [CrossRef] [PubMed]
53. Esmaili, S.; Xu, A.; George, J. The multifaceted and controversial immunometabolic actions of adiponectin. *Trends Endocrinol. Metab.* **2014**, *25*, 444–451. [CrossRef] [PubMed]
54. Bloemer, J.; Pinky, P.D.; Govindarajulu, M.; Hong, H.; Judd, R.; Amin, R.H.; Moore, T.; Dhanasekaran, M.; Reed, M.N.; Suppiramaniam, V. Role of adiponectin in central nervous system disorders. *Neural. Plast.* **2018**, *2018*, 4593530. [CrossRef]
55. Bartesaghi, S.; Radi, R. Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration. *Redox Biol.* **2018**, *14*, 618–625. [CrossRef]
56. Liem, R.K.H.; Messing, A. Dysfunctions of neuronal and glial intermediate filaments in disease. *J. Clin. Investig.* **2009**, *119*, 1814–1824. [CrossRef]
57. Kowiański, P.; Lietzau, G.; Czuba, E.; Waśkow, M.; Steliga, A.; Moryś, J. BDNF: A Key Factor with Multipotent Impact on Brain Signaling and Synaptic Plasticity. *Cell. Mol. Neurobiol.* **2018**, *38*, 579–593. [CrossRef]
58. Glerup, S.; Bolcho, U.; Mølgaard, S.; Bøggild, S.; Vaegter, C.B.; Smith, A.H.; Nieto-Gonzalez, J.L.; Ovesen, P.L.; Pedersen, L.F.; Fjorback, A.N.; et al. SorCS2 is required for BDNF-dependent plasticity in the hippocampus. *Mol. Psychiatry* **2016**, *21*, 1740–1751. [CrossRef]
59. Meng, Q.; Ying, Z.; Noble, E.; Zhao, Y.; Agrawal, R.; Mikhail, A.; Zhuang, Y.; Tyagi, E.; Zhang, Q.; Lee, J.H.; et al. Systems nutrigenomics reveals brain gene networks linking metabolic and brain disorders. *EBioMedicine* **2016**, *7*, 157–166. [CrossRef]
60. Zhang, D.M.; Jiao, R.Q.; Kong, L.D. High Dietary Fructose: Direct or Indirect Dangerous Factors Disturbing Tissue and Organ Functions. *Nutrients* **2017**, *9*, 335. [CrossRef]
61. Sindhunata, D.P.; Meijnikman, A.S.; Gerdes, V.E.A.; Nieuwdorp, M. Dietary fructose as a metabolic risk factor. *Am. J. Physiol. Cell Physiol.* **2022**, *323*, C847–C856. [CrossRef] [PubMed]
62. Ochoa, M.; Lallès, J.P.; Malbert, C.H.; Val-Laillet, D. Dietary sugars: Their detection by the gut-brain axis and their peripheral and central effects in health and diseases. *Eur. J. Nutr.* **2015**, *54*, 1–24. [CrossRef] [PubMed]
63. Miller, C.C.; Martin, R.J.; Whitney, M.L.; Edwards, G.L. Intracerebroventricular injection of fructose stimulates feeding in rats. *Nutr Neurosci.* **2002**, *5*, 359–362. [CrossRef] [PubMed]
64. Cha, S.H.; Wolfgang, M.; Tokutake, Y.; Chohnan, S.; Lane, M.D. Differential effects of central fructose and glucose on hypothalamic malonyl-CoA and food intake. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 16871–16875. [CrossRef]
65. Havel, P.J. Glucose but not fructose infusion increases circulating leptin in proportion to adipose stores in Rhesus monkeys. *Exp. Clin. Endocrinol. Diabetes* **1997**, *105*, 37–38. [CrossRef]
66. Teff, K.L.; Elliott, S.S.; Tschop, M.; Kieffer, T.J.; Rader, D.; Heiman, M.; Townsend, R.R.; Keim, N.L.; D'Alessio, D.; Havel, P.J. Dietary fructose reduces circulating insulin and leptin, attenuates postprandial suppression of ghrelin, and increases triglycerides in women. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 2963–2972. [CrossRef]
67. Figlewicz, D.P.; Bennett, J.; Evans, S.B.; Kaiyala, K.; Sipols, A.J.; Benoit, S.C. Intraventricular insulin and leptin reverse place preference conditioned with high-fat diet in rats. *Behav. Neurosci.* **2004**, *118*, 479–487. [CrossRef]
68. Ochoa, M.; Malbert, C.H.; Lallès, J.P.; Bobillier, E.; Val-Laillet, D. Effects of chronic intake of starch-, glucose- and fructose containing diets on eating behaviour in adult minipigs. *Appl. Anim. Behav. Sci.* **2014**, *157*, 61–71. [CrossRef]
69. Shapiro, A.; Mu, W.; Roncal, C.; Cheng, K.Y.; Johnson, R.J.; Scarpace, P.J. Fructose-induced leptin resistance exacerbates weight gain in response to subsequent high-fat feeding. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2008**, *295*, R1370–R1375. [CrossRef]

70. Bursać, B.N.; Vasiljević, A.D.; Nestorović, N.M.; Veličković, N.A.; Vojnović Milutinović, D.D.; Matić, G.M.; Djordjevic, A.D. High-fructose diet leads to visceral adiposity and hypothalamic leptin resistance in male rats-Do glucocorticoids play a role? *J. Nutr. Biochem.* **2014**, *25*, 446–455. [CrossRef]
71. Lindqvist, A.; Baelemans, A.; Erlanson-Albertsson, C. Effects of sucrose, glucose and fructose on peripheral and central appetite signals. *Regul. Pept.* **2008**, *150*, 26–32. [CrossRef] [PubMed]
72. Pan, Y.; Kong, L.D. High fructose diet-induced metabolic syndrome: Pathophysiological mechanism and treatment by traditional Chinese medicine. *Pharmacol. Res.* **2018**, *130*, 438–450. [CrossRef] [PubMed]
73. Papa, S.; Martino, P.L.; Capitanio, G.; Gaballo, A.; De Rasmo, D.; Signorile, A.; Petruzzella, V. The oxidative phosphorylation system in mammalian mitochondria. *Adv. Exp. Med. Biol.* **2012**, *942*, 3–37. [CrossRef]
74. Larsen, S.; Nielsen, J.; Hansen, C.N.; Nielsen, L.B.; Wibrand, F.; Stride, N.; Schroder, H.D.; Boushel, R.; Helge, J.W.; Dela, F.; et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol.* **2012**, *590*, 3349–3360. [CrossRef]
75. Zhou, Z.; Hagopian, K.; López-Domínguez, J.A.; Kim, K.; Jasoliya, M.; Roberts, M.N.; Cortopassi, G.A.; Showalter, M.R.; Roberts, B.S.; González-Reyes, J.A.; et al. A ketogenic diet impacts markers of mitochondrial mass in a tissue specific manner in aged mice. *Aging* **2021**, *13*, 7914–7930. [CrossRef] [PubMed]
76. Narendra, D.P.; Jin, S.M.; Tanaka, A.; Suen, D.F.; Gautier, C.A.; Shen, J.; Cookson, M.R.; Youle, R.J. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* **2010**, *8*, e1000298. [CrossRef]
77. Shoshan-Barmatz, V.; Maldonado, E.N.; Krelin, Y. Vdac1 at the crossroads of cell metabolism, apoptosis and cell stress. *Cell Stress* **2017**, *1*, 11–36. [CrossRef]
78. Wallace, D.C. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: A dawn for evolutionary medicine. *Annu. Rev. Genet.* **2005**, *39*, 359–407. [CrossRef]
79. Golpich, M.; Amini, E.; Mohamed, Z.; Azman Ali, R.; Mohamed Ibrahim, N.; Ahmadiani, A. Mitochondrial dysfunction and biogenesis in neurodegenerative diseases: Pathogenesis and treatment. *CNS Neurosci. Ther.* **2017**, *23*, 5–22. [CrossRef]
80. Albensi, B.C. Dysfunction of mitochondria: Implications for Alzheimer's disease. *Int. Rev. Neurobiol.* **2019**, *145*, 13–27. [CrossRef]
81. Bianchi, R.; Kastrisianaki, E.; Giambanco, I.; Donato, R. S100B protein stimulates microglia migration via RAGE-dependent up-regulation of chemokine expression and release. *J. Biol. Chem.* **2011**, *286*, 7214–7226. [CrossRef] [PubMed]
82. Xu, J.; Wang, H.; Won, S.J.; Basu, J.; Kapfhamer, D.; Swanson, R.A. Microglial activation induced by the alarmin S100B is regulated by poly (ADP-ribose) polymerase-1. *Glia* **2016**, *64*, 1869–1878. [CrossRef]
83. Rahman, M.H.; Kim, M.S.; Lee, I.K.; Yu, R.; Suk, K. Corrigendum: Interglial Crosstalk in Obesity-Induced Hypothalamic Inflammation. *Front. Neurosci.* **2019**, *13*, 3. [CrossRef] [PubMed]
84. Rahman, M.H.; Bhusal, A.; Lee, W.H.; Lee, I.K.; Suk, K. Hypothalamic inflammation and malfunctioning glia in the pathophysiology of obesity and diabetes: Translational significance. *Biochem. Pharmacol.* **2018**, *153*, 123–133. [CrossRef] [PubMed]
85. Spezani, R.; da Silva, R.R.; Martins, F.F.; de Souza Marinho, T.; Aguila, M.B.; Mandarim-de-Lacerda, C.A. Intermittent fasting, adipokines, insulin sensitivity, and hypothalamic neuropeptides in a dietary overload with high-fat or high-fructose diet in mice. *J. Nutr. Biochem.* **2020**, *83*, 108419. [CrossRef] [PubMed]
86. Raja Gopal Reddy, M.; Jeyakumar, S.M.; Vajreswari, A. Consumption of vitamin A-deficient diet elevates endoplasmic reticulum stress marker and suppresses high fructose-induced orexigenic gene expression in the brain of male Wistar rats. *Nutr. Neurosci.* **2022**, *25*, 1872–1880. [CrossRef] [PubMed]
87. Mazzoli, A.; Gatto, C.; Crescenzo, R.; Cigliano, L.; Iossa, S. Prolonged Changes in Hepatic Mitochondrial Activity and Insulin Sensitivity by High Fructose Intake in Adolescent Rats. *Nutrients* **2021**, *13*, 1370. [CrossRef] [PubMed]
88. Samant, N.P.; Gupta, G.L. Adiponectin: A potential target for obesity-associated Alzheimer's disease. *Metab. Brain Dis.* **2021**, *36*, 1565–1572. [CrossRef]
89. Chan, K.-H.; Lam, K.S.-L.; Cheng, O.-Y.; Kwan, J.S.-C.; Ho, P.W.-L.; Cheng, K.K.-Y.; Chung, S.K.; Ho, J.W.-M.; Guo, V.Y.; Xu, A. Adiponectin is protective against oxidative stress induced cytotoxicity in amyloid-beta neurotoxicity. *PLoS ONE* **2012**, *7*, e52354. [CrossRef]
90. Ng, R.C.-L.; Chan, K.-H. Potential Neuroprotective Effects of Adiponectin in Alzheimer's Disease. *Int. J. Mol. Sci.* **2017**, *18*, 592. [CrossRef]
91. Lee, H.; Tu, T.H.; Park, B.S.; Yang, S.; Kim, J.G. Adiponectin Reverses the Hypothalamic Microglial Inflammation during Short-Term Exposure to Fat-Rich Diet. *Int. J. Mol. Sci.* **2019**, *20*, 5738. [CrossRef] [PubMed]
92. Koch, C.E.; Lowe, C.; Legler, K.; Benzler, J.; Boucsein, A.; Böttiger, G.; Grattan, D.R.; Williams, L.M.; Tups, A.; Grattan, D. Central Adiponectin Acutely Improves Glucose Tolerance in Male Mice. *Endocrinology* **2014**, *155*, 1806–1816. [CrossRef] [PubMed]
93. Kaminska, B.; Czerwinska, J.; Bogacka, I.; Chojnowska, K.; Smolinska, N.; Dobrzyn, K.; Kiezuna, M.; Zaobidna, E.; Myszczynski, K.; Nowakowski, J.J.; et al. Sex- and season-dependent differences in the expression of adiponectin and adiponectin receptors (AdipoR1 and AdipoR2) in the hypothalamic-pituitaryadrenal axis of the Eurasian beaver (*Castor fiber* L.). *Gen. Comp. Endocrinol.* **2020**, *298*, 113575. [CrossRef] [PubMed]
94. Kaminski, T.; Smolinska, N.; Maleszka, A.; Kiezun, M.; Dobrzyn, K.; Czerwinska, J.; Szeszko, K.; Nitkiewicz, A. Expression of Adiponectin and its Receptors in the Porcine Hypothalamus During the Oestrous Cycle. *Reprod. Domest. Anim.* **2014**, *49*, 378–386. [CrossRef]

95. Abgrall, A.; Poizat, G.; Prevost, M.; Riffault, L.; De La Barrera, L.; Hanine, R.; Djordjevic, K.; Benomar, Y.; Taouis, M. Evidence for the Neuronal Expression and Secretion of Adiponectin. *Cells* **2022**, *11*, 2725. [CrossRef]
96. Thundyil, J.; Pavlovski, D.; Sobey, C.G.; Arumugam, T.V. Adiponectin receptor signalling in the brain. *Br. J. Pharm.* **2012**, *165*, 313–327. [CrossRef]
97. Qiu, G.; Wan, R.; Hu, J.; Mattson, M.P.; Spangler, E.; Liu, S.; Yau, S.S.Y.; Lee, T.M.C.; Gleichmann, M.; Ingram, D.K.; et al. Adiponectin protects rat hippocampal neurons against excitotoxicity. *Age* **2011**, *33*, 155–165. [CrossRef]
98. Yau, S.Y.; Li, A.; Hoo, R.L.C.; Ching, Y.P.; Christie, B.R.; Lee, T.M.C.; Xu, A.; So, K.-F. Physical exercise-induced hippocampal neurogenesis and antidepressant effects are mediated by the adipocyte hormone adiponectin. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 15810–15815. [CrossRef]
99. Zhu, Q.; Couillard-Despres, S.; Julien, J.P. Delayed maturation of regenerating myelinated axons in mice lacking neurofilaments. *Exp. Neurol.* **1997**, *148*, 299–316. [CrossRef]
100. Yum, S.W.; Zhang, J.; Mo, K.; Li, J.; Scherer, S.S. A novel recessive Nefl mutation causes a severe, early-onset axonal neuropathy. *Ann. Neurol.* **2009**, *66*, 759–770. [CrossRef]
101. Yuan, A.; Sershen, H.; Veeranna; Basavarajappa, B.S.; Kumar, A.; Hashim, A.; Berg, M.; Lee, J.-H.; Sato, Y.; Rao, M.V.; et al. Neurofilament subunits are integral components of synapses and modulate neurotransmission and behavior in vivo. *Mol. Psychiatry* **2015**, *20*, 986–994. [CrossRef] [PubMed]
102. Yuan, A.; Sershen, H.; Veeranna; Basavarajappa, B.S.; Kumar, A.; Hashim, A.; Berg, M.; Lee, J.-H.; Sato, Y.; Rao, M.V.; et al. Functions of neurofilaments in synapses. *Mol. Psychiatry* **2015**, *20*, 915. [CrossRef] [PubMed]
103. Yuan, A.; Rao, M.V.; Veeranna; Nixon, R.A. Neurofilaments and Neurofilament Proteins in Health and Disease. *Cold Spring Harb. Perspect. Biol.* **2017**, *9*, a018309. [CrossRef] [PubMed]
104. Puram, S.V.; Kim, A.H.; Ikeuchi, Y.; Wilson-Grady, J.T.; Merdes, A.; Gygi, S.P.; Azad, B.A. Unique CaMKIIβ Signaling Pathway at the Centrosome Regulates Dendrite Patterning in the Brain. *Nat. Neurosci.* **2011**, *14*, 973–983. [CrossRef] [PubMed]
105. Sugawara, T.; Hisatsune, C.; Miyamoto, H.; Ogawa, N.; Mikoshiba, K. Regulation of spinogenesis in mature Purkinje cells via mGluR/PKC-mediated phosphorylation of CaMKII. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E5256–E5265. [CrossRef]
106. Sałaciak, K.; Koszałka, A.; Żmudzka, E.; Pytka, K. The Calcium/Calmodulin-Dependent Kinases II and IV as Therapeutic Targets in Neurodegenerative and Neuropsychiatric Disorders. *Int. J. Mol. Sci.* **2021**, *22*, 4307. [CrossRef]
107. Spagnuolo, M.S.; Mazzoli, A.; Nazzaro, M.; Troise, A.D.; Gatto, C.; Tonini, C.; Colardo, M.; Segatto, M.; Scaloni, A.; Pallottini, V.; et al. Long-Lasting Impact of Sugar Intake on Neurotrophins and Neurotransmitters from Adolescence to Young Adulthood in Rat Frontal Cortex. *Mol. Neurobiol.* **2022**. [CrossRef]
108. Ma, Q.; Yang, J.; Milner, T.A.; Vonsattel, J.G.; Palko, M.E.; Tessarollo, L.; Hempstead, B.L. SorCS2-mediated NR2A trafficking regulates motor deficits in Huntington's disease. *JCI Insight* **2017**, *2*, e88995. [CrossRef]
109. Yoshii, A.; Constantine-Paton, M. BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. *Nat. Neurosci.* **2007**, *10*, 702–711. [CrossRef]
110. Hu, X.; Ballo, L.; Pietila, L.; Viesselmann, C.; Ballweg, J.; Lumbard, D.; Stevenson, M.; Merriam, E.; Dent, E.W. BDNF-induced increase of PSD-95 in dendritic spines requires dynamic microtubule invasions. *J. Neurosci.* **2011**, *31*, 15597–15603. [CrossRef]
111. Cheng, A.; Wan, R.; Yang, J.L.; Kamimura, N.; Son, T.G.; Ouyang, X.; Luo, Y.; Okun, E.; Mattson, M.P. Involvement of PGC-1α in the formation and maintenance of neuronal dendritic spines. *Nat. Commun.* **2012**, *3*, 1250. [CrossRef] [PubMed]

SUPPLEMENTARY MATERIAL

Table S1. Identification details of differentially represented proteins

Reported are the information on protein false discovery rate (FDR) confidence, protein accession, description, gene name, exp. q-value, sum posterior error probability (PEP) score, sequence coverage (%), number of identified peptides, peptide spectrum matches (PSMs), number of identified unique peptides, number of amino acids, molecular mass, pI, Mascot identification score values, Razor Peptides, Abundance Ratio, Abundance Ratio (log2), Abundance Ratio P-Value, Abundance Ratio Adj. P-Value, Abundances (Grouped), Abundances (Grouped) CV [%].

FDR Confidence: Combined	Accession	Description	Gene name	Exp. q-value: Combined	Sum PEP Score	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	# AAs	MW [kDa]	calc. pI	Mascot: Mascot	Peptides (by Search Engine):	# Razor Peptides	Abundance Ratio: F/C	Abundance Ratio: FR / CR	Abundance Ratio (log2): F/C	Abundance Ratio (log2): FR / CR)	Abundance Ratio P-Value: F / C	Abundance Ratio P-Value: FR / CR	Abundance Ratio Adj. P-Value: F / C	Abundance Ratio Adj. P-Value: FR / CR	Abundances (Grouped): C	Abundances (Grouped): CR	Abundances (Grouped): F	Abundances (Grouped): FR	Abundances (Grouped) CV [%]: C	Abundances (Grouped) CV [%]: CR	Abundances (Grouped) CV [%]: F	Abundances (Grouped) CV [%]: FR
High	D4AEC0	Histone H2A OS=Rattus norvegicus	H2afv	0	7,211	15	2	7	1	128	13,5	10,58	137	2	0	1,663	1,153	0,73	0,21	0,000144367	0,103786106	0,098440031	1	78,3	88,9	130,3	102,5	7,7	4,49	7,77	2,82
High	P01186	Vasopressin-neurophysin 2-copeptin OS=Rattus norvegicus	Avp	0	32,91	46	5	17	5	168	17,9	6,42	402	5	0	1,253	0,999	0,33	0	0,017325092	0,999878548	1	1	88,9	100	111,3	99,8	3,9	2,56	1,48	2,22
High	P04631	Protein S100-B OS=Rattus norvegicus	S100b	0	47,209	40	4	98	4	92	10,7	4,55	2444	4	0	1,25	0,908	0,32	-0,14	0,022714908	0,362054366	1	1	79,4	115,9	99,3	105,3	1,36	0,94	0,74	1,28
High	G3V9G3	Calcium/calmodulin-dependent protein kinase type II subunit beta	Camk2b	0	106,09	44	17	81	9	542	60,4	7,28	2077	17	8	0,828	0,938	-0,27	-0,09	0,042986627	0,667514111	1	1	104	108,3	86,1	101,6	0,96	1,05	0,58	0,73
High	D3ZW03	Sortilin-related VPS10 domain-containing receptor 2	Sorcs2	0	5,603	2	2	2	2	1158	128,7	7,37	46	2	0	0,812	1,033	-0,3	0,05	0,011712893	0,779640991	1	1	110,1	98,7	89,4	101,9	3,99	5,18	0,89	0,07
High	Q66HF1	NADH-ubiquinone oxidoreductase	Ndufs1	0	160,73	50	25	105	25	727	79,4	5,9	2687	25	0	0,792	0,904	-0,34	-0,15	3,59956E-06	0,002126498	0,01631957	1	100,9	115,1	79,9	104,1	1,1	0,9	1,73	0,46
High	G3V8F1	Transporter OS=Rattus norvegicus	S1c6a7	0	6,307	3	2	5	2	637	71	6,61	116	2	0	0,787	1,035	-0,35	0,05	0,009751426	0,792525336	1	1	116,2	94,6	91,4	97,8	2,67	0,7	6,43	2,17
High	B5DEH6	Erlin-2 OS=Rattus norvegicus OX	Erlin2	0	22,374	17	6	12	6	339	37,7	5,74	174	6	0	0,787	0,929	-0,35	-0,11	0,032382357	0,661928666	1	1	112,8	102,9	88,7	95,6	1,79	1	2,89	0,34
High	M0RCX4	4a-hydroxytetrahydrobiopterin dehydratase	Pcbd2	0,001	4,508	11	2	5	1	127	14,1	9,2	68	2	0	0,778	0,92	-0,36	-0,12	0,040529395	0,646961752	1	1	104,9	111,2	81,6	102,3	1,35	8,09	9,64	0,79
High	D3Z9R6	ATP synthase subunit ATP5MJ, mitochondrial	Atp5mj	0	6,537	30	2	4	2	60	6,9	9,92	63	2	0	0,776	0,969	-0,37	-0,05	0,000198956	0,247206181	0,120589251	1	99,1	113,8	76,9	110,2	0,86	1,01	1,89	1,59
High	P19527	Neurofilament light polypeptide OX	Nefl	0	134,43	55	26	152	23	542	61,3	4,65	2913	26	0	0,761	0,907	-0,39	-0,14	0,001265901	0,191562101	0,531191734	1	99,1	118,2	75,4	107,2	1,25	1,99	2,88	0,92
High	B2RYW0	Complex I-B22 OS=Rattus norvegicus	Ndufb9	0	7,726	20	3	12	3	179	21,9	8,07	301	3	0	0,737	0,903	-0,44	-0,15	5,9624E-05	0,03306631	0,070895365	1	99,6	119,3	73,5	107,7	1,58	2,07	1,46	1,36
High	D3ZD05	Cytochrome c oxidase subunit	Cox6b1	0	20,381	62	5	24	5	86	10,1	8,72	574	5	0	0,729	0,913	-0,46	-0,13	3,43772E-05	0,132018961	0,062509183	1	98,3	120,2	71,6	109,8	1,67	1,53	6,13	2,06
High	D4A0T0	Complex I-PDSW OS=Rattus norvegicus	Ndufb10	0	21,078	26	4	16	4	176	20,8	7,69	370	4	0	0,726	0,886	-0,46	-0,17	0,019276616	0,425969694	1	1	101,8	118,9	73,9	105,3	0,53	1,4	5,21	1,66
High	P12839	Neurofilament medium polypeptide	Nefm	0	149,86	43	35	153	31	846	95,7	4,79	3047	35	5	0,709	0,903	-0,5	-0,15	0,029305965	0,734079869	1	1	102,4	118,2	72,6	106,8	0,83	0,52	1,88	0,61
High	P23565	Alpha-internexin OS=Rattus norvegicus	Ina	0	143,31	55	27	217	2	505	56,1	5,22	5419	27	21	0,688	0,88	-0,54	-0,18	5,98334E-06	0,009589728	0,01631957	1	99,7	123,3	68,6	108,4	0,85	0,86	1,76	1,01
High	A0A0G2	Voltage-dependent anion-selective	Vdac3	0	69,843	54	12	39	11	283	30,8	8,59	1038	12	1	0,563	0,799	-0,83	-0,32	0,012661965	0,4642522	1	1	100,9	134,7	56,8	107,7	1,25	2,29	8,24	1,3
High	Q9Z2L0	Voltage-dependent anion-selective	Vdac1	0	123,83	78	16	100	16	283	30,7	8,54	2838	16	0	0,561	0,819	-0,83	-0,29	6,4982E-05	0,052250196	0,070895365	1	103,2	131,4	57,9	107,6	1,46	0,98	4,97	0,25
High	P81155	Voltage-dependent anion-selective	Vdac2	0	64,382	52	11	51	10	295	31,7	7,49	1111	11	0	0,534	0,816	-0,9	-0,29	0,005847761	0,455456088	1	1	102,1	133,9	54,6	109,4	0,72	1,77	7,31	1
High	A1A5N6	Hyaluronan and proteoglycan link	Heplnl	0	9,701	8	2	6	2	354	40,2	7,75	190	2	0	0,909	1,306	-0,14	0,39	0,027758747	1,61383E-05	1	0,022008371	96,7	93,4	87,8	122,1	1,99	4,19	0,17	1,67
High	D4AAJ1	Dmx-like 1 OS=Rattus norvegicus	Dmxl1	0	8,97	1	3	4	1	3017	336,8	6,44	91	3	0	1,105	1,327	0,14	0,41	0,296211629	0,013904761	1	1	90,9	89,6	100,3	119	3,82	2,01	3,26	7,18
High	A0A0G2	Transgelin OS=Rattus norvegicus	Tagln	0	39,171	40	9	20	8	207	23,3	8,66	488	9	0	1,046	1,48	0,07	0,57	0,933241414	0,022808411	1	1	100,2	78,6	104,9	116,3	1,76	3,19	3,51	4,26
High	A0A140	Protein kinase C delta type OS=Rattus	Prkcd	0	5,995	3	2	6	1	673	77,5	7,83	97	2	0	0,912	1,281	-0,13	0,36	0,455960047	0,039963264	1	1	108,9	84,1	99,2	107,8	1,17	1,18	0,1	0,36

Figure S1. Proteomic analysis of hypothalamic tissues

Volcano plot representing differentially expressed proteins in hypothalamic tissues isolated from fructose-fed adolescents versus control adolescent rats. Over- and down-represented proteins (Log2FC > 0.26, $p < 0.05$) are reported in red and green area, respectively.

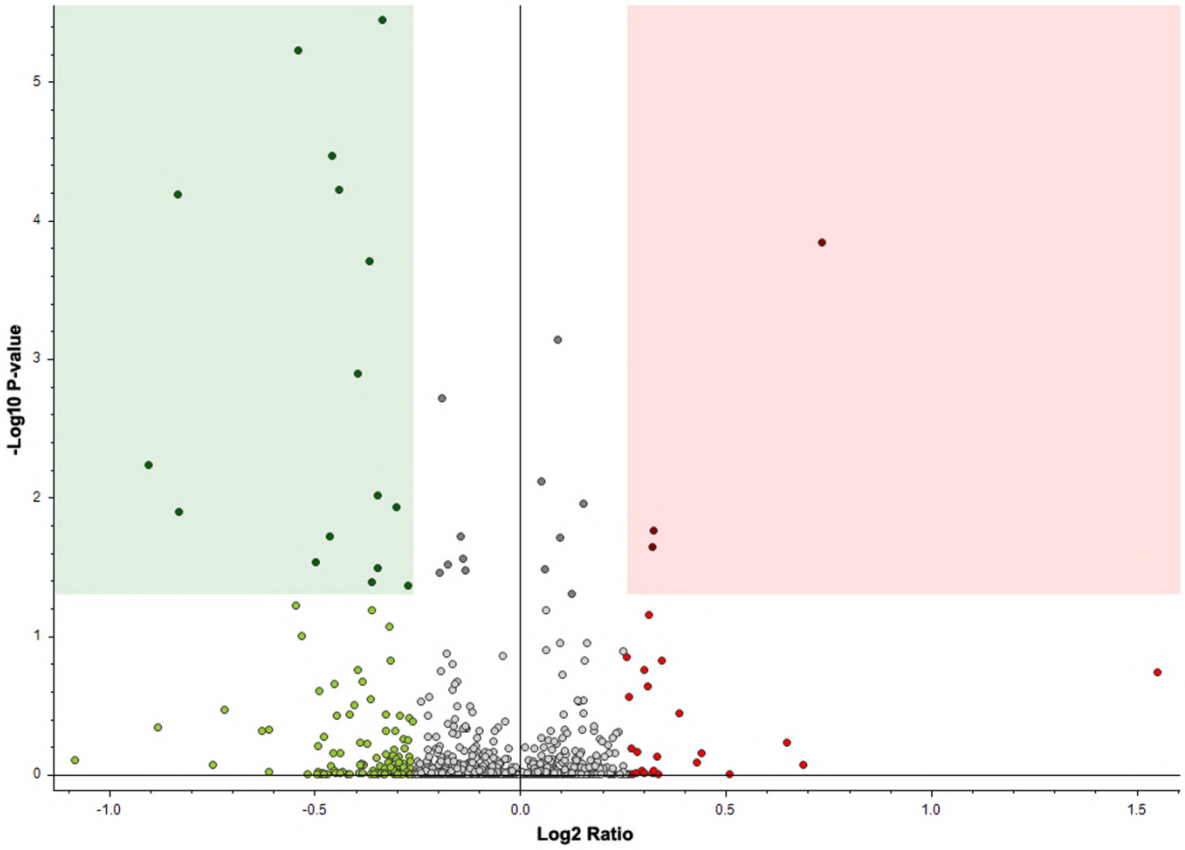

Figure S2. Heat-map hierarchical cluster analysis

Heat-map hierarchical cluster analysis of proteins differentially represented in hypothalamic tissues of fructose-fed adolescent (F, orange) and control adolescent (C, light blue) rats, young-adult fructose-rescued (FR, green) and young-adult control rescued (CR, red) rats. Dendrograms on the left report the protein grouping and distance between protein classes, while dendrograms on the top report the sample grouping. Normalized area ranges and Eculidean distance were used; scaled expression values of each range are plotted in green to red through black color scale.

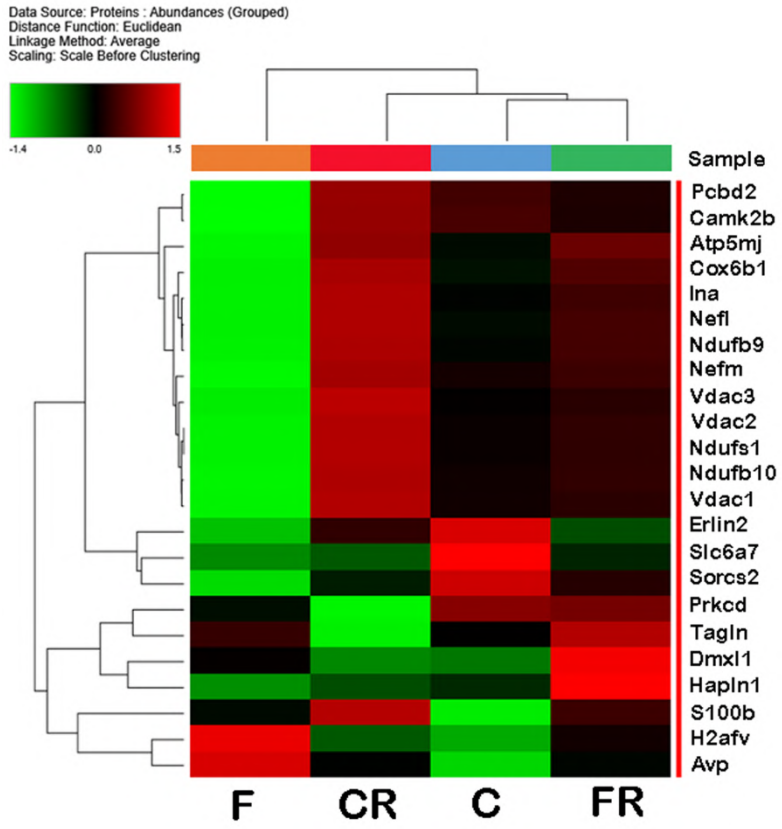

Figure S3. Adiponectin level in plasma

Adiponectin concentration was measured in plasma samples of control adolescent (C), fructose-fed adolescent (F), young-adult control rescued (CR), and young-adult fructose-rescued (FR) rats.
All the plasma samples were adjusted to protein concentration of 10 µg/µL and 5 µL of each sample were analyzed by 12.5 % SDS PAGE and western blotting. Immunocomplexes were detected by rabbit anti-adiponectin and GAR-HRP IgGs. Densitometric data are reported as means ± SEM of 6 different rats from each experimental group. ** $p < 0.01$ vs C; # $p < 0.05$ vs CR (one-way Anova followed by Bonferroni post-test).

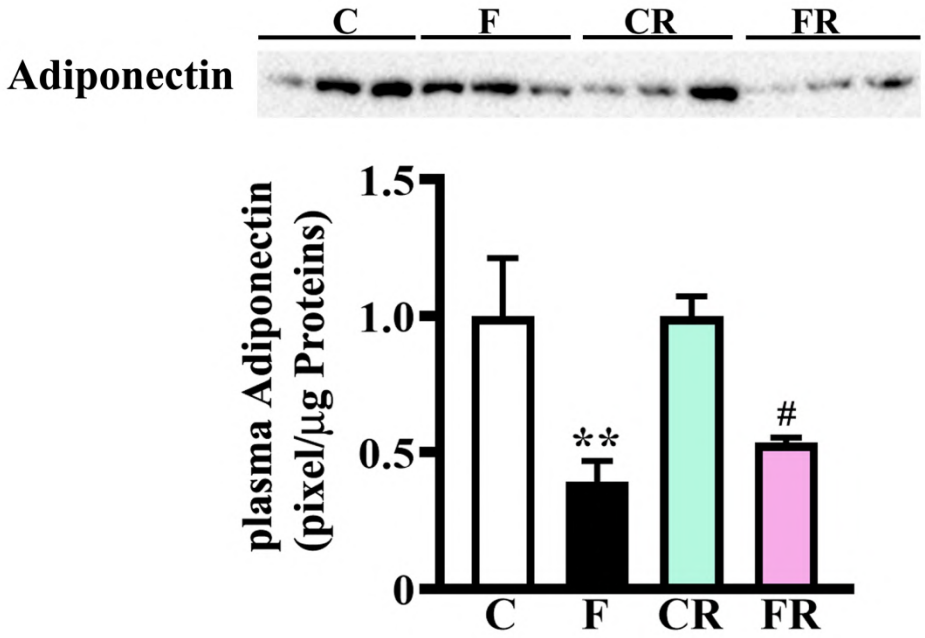

Figure S3

Chapter 5

Concluding remarks

Concluding remarks

Fructose consumption, which drastically increased during the nineteenth and twentieth centuries due to the widespread use of HFCS, has long been known to induce overweigh, obesity and inflammation, as well as dyslipidemia, insulin resistance and related metabolic diseases. Emerging data also indicates that high fructose diets have a profound impact on brain function (Spagnuolo et al.,2020). The research group where I did my PhD already showed that fructose-rich diet can affect brain metabolism in both young and adult animal models (Cigliano et al.,2018; Spagnuolo et al.,2020). A major aim of the PhD research plan was to further clarify the effect of a short-term fructose-rich diet on the brain, throughout adolescent development, and the possible persistence of sugar-induced changes until young adulthood, after returning to a balanced diet. In particularl, attention was focused on both hippocampus and the frontal cortex, brain regions involved in learning and memory, particularly vulnerable to dietary and metabolic insults (Hsu et al.,2014; Hannapel et al.,2017). A further objective of this PhD work was to clarify the effect of the fructose-rich diet also on hypothalamus, a brain area playing a key role in maintaining whole-body homeostasis (Elmquist et al.,2005; Marty et al.,2007).

Overall, the results here reported indicate that a diet rich in fructose, even if accomplished for short period, induces neuroinflammation and oxidative stress, and highly affects the level of specific markers of brain function and plasticity, determining alterations that may anticipate or predispose to dysfunctions in the long-term period. Unlike what we observed in the frontal cortex of fructose-fed rats (Spagnuolo et al.,2022), the fructose-driven changes detected in hypothalamus and hippocampus were almost fully rescued after switching to the control diet. Conversely, some of the alterations in the frontal cortex (BDNF, CML, CEL, acetylcholinesterase activity, dysregulation of neurotransmitter levels) persisted after switching to the control diet. These resuls indicate that fructose can have a different impact depending on the brain region. The greater sensitivity of the frontal cortex could be linked to the fact that, unlike the other two areas examined, it continues its maturation until adulthood (Giedd et al.,1999; Casey et al.,2008; Mills et al.,2014) and this could make it more susceptible to permanent damage caused by incorrect nutrition during adolescence. Further studies are obviously needed to clarify the mechanisms underlying the different response to the sugar.

The reasons for the persistence of the alterations found in the frontal cortex can be multifactorial. With reference to BDNF reduction, a fascinating hypothesis, supported by a recently published paper, calls into question possible epigenetic modifications (Kageyama et al.,2022). In fact, it was shown that HFCS consumption reduced brain Bdnf mRNA and protein expressions in childhood and adolescence and pyrosequencing assays revealed increased DNA

methylation at the Bdnf promoter in childhood and adolescence, thus suggesting that the decrease of BDNF may be due to hypermethylation of the promoter regions. It should be noted that this phenomenon was observed only in childhood and adolescence but not in adults, thus suggesting the sensitivity of the brain to fructose may vary with age (Kageyama et al.,2022). Also, it has been shown that excess maternal fructose consumption may also modify BDNF DNA methylation, thereby suppressing transcriptional activity in offspring. This, in turn, may lead to impaired cognitive performance and reduced adult hippocampal neurogenesis (Yamazaki et al.,2018).

A further factor that may play a role in the fructose-driven effects on brain is the microbiota reshaping induced by the sugar. Different line of evidence indicates that gut microbiota plays an important role in gut-brain axis and behavior by producing metabolites, hormones and immune factors that can influence the brain (Trzeciak et al.,2021). Fructose affects microbiota composition and abundance that has been reported to be associated with metabolic dysregulation and inflammation in hypothalamus, liver and adipose tissues (Ahn et al.,2020). Although not reported in this PhD thesis, metagenomic analysis of gut microbiota and short chain fatty acids levels (faeces and plasma) were investigated and reshaping of gut microbiota and altered content of short chain fatty acids was elicited by the fructose diet (Mazzoli et al., 2022). These changes were abolished by switching back to control diet, but it cannot be excluded that fructose-induced changes in gut microbiota are involved in the onset of metabolic syndrome but this metabolic imbalance, once started, likely triggers in turn other alterations directly (i.e.bacterial-derived metabolites) or not directly linked to gut microbiota that persist beyond the changes in dietary treatment, as it was showed in the frontal cortex in this PhD research work.

The research area related to the impact of fructose on brain function is certainly less explored than those devoted to the effects of this sugar on other organs. Hence, a challenge for the near future could be to fully elucidate the direct effects of different concentrations of fructose on brain cells, as well as the real amount of this sugar reaching the brain compartment following different fructose intake. Furthermore, as emerging data indicate that fructose affects microbiota composition and abundance that are associated with metabolic dysregulation and select for pro-inflammatory phenotypes in brain (Ahn et al.,2020), liver (Mazzoli et al.,2021) and adipose tissues (Ahn et al.,2020), a further goal is to determine whether the administration of probiotics can limit or prevent the alterations induced by the fructose-rich diet.

The research data collected so far point out that targeted dietary interventions, as well as specific policies, are necessary to reduce the consumption of refined sugars worldwide, not only to

prevent overweight and metabolic syndrome but, importantly, to promote brain health in adolescent and during growth. The here shown results delineate a picture reflecting a very harmful situation, considering the increasing consumption of fructose especially in the young populations, that are posed at risk of experiencing stable metabolic modifications that can certainly impact on their brain status later in the adulthood.

References

1. I.S. Ahn, J.M. Lang, C.A. Olson, G. Diamante, G. Zhang, Z. Ying, H.R. Byun, I. Cely, J. Ding, P. Cohn, I. Kurtz, F. Gomez-Pinilla, A.J. Lusis, E.Y. Hsiao, X. Yang, Host genetic background and gut microbiota contribute to differential metabolic responses to fructose consumption in mice, J. Nutr. 150 (10) (2020) 2716–2728.

2. Casey BJ, Jones RM, Hare TA (2008) The adolescent brain. Ann N Y Acad Sci 1124:111-126.

3. Cigliano, L.; Spagnuolo, M.S.; Crescenzo, R.; Cancelliere, R.; Iannotta, L.; Mazzoli, A.; Liverini, G.; Iossa, S. Short-Term Fructose Feeding Induces Inflammation and Oxidative Stress in the Hippocampus of Young and Adult Rats. Mol. Neurobiol. 2018, 55, 2869–2883.

4. Elmquist, J.K.; Coppari, R.; Balthasar, N.; Ichinose, M.; Lowell, B.B. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. J. Comp. Neurol. 2005, 493(1), 63-71; DOI:10.1002/cne.20786.

5. Giedd JN, Blumenthal J, Jeffries NO, Castellanos FX, Liu H, Zijdenbos A, Paus T, Evans AC, Rapoport JL (1999) Brain development during childhood and adolescence: a longitudinal MRI study. Nat Neurosci 2(10):861-863.

6. Hannapel, R.C.; Henderson, Y.H.; Nalloor, R.; Vazdarjanova, A.; Parent, M.B. Ventral hippocampal neurons inhibit postprandial energy intake. Hippocampus 2017, 27, 274–284

7. Hsu, T.M.; Kanoski, S.E. Blood-brain barrier disruption: Mechanistic links between Western diet consumption and dementia. Front. Aging Neurosci. 2014, 6, 88.

8. Kageyama I, Yamada H, Munetsuna E, Yamazaki M, Ando Y, et al. (2022) Differential effects of excess high-fructose corn syrup on the DNA methylation of hippocampal neurotrophic factor in childhood and adolescence. PLOS ONE 17(6): e0270144.

9. Marty, N.; Dallaporta, M.; Thorens, B. Brain glucose sensing, counterregulation, and energy homeostasis. Physiology (Bethesda). 2007, 22, 241-251; DOI:10.1152/physiol.00010.2007.

10. Mazzoli A, Gatto C, Crescenzo R, Spagnuolo MS, Nazzaro M, Iossa S, Cigliano L. Gut and liver metabolic responses to dietary fructose - are they reversible or persistent after switching to a healthy diet? Food Funct. 2021 Aug 21;12(16):7557-7568. doi: 10.1039/d1fo00983d. Epub 2021 Jul 21. PMID: 34286786

11. Mazzoli A, Porzio AD, Gatto C, Crescenzo R, Nazzaro M, Spagnuolo MS, Baccigalupi L, Ricca E, Amoresano A, Fontanarosa C, Bernacchioni C, Donati C, Iossa S, Cigliano L. Skeletal muscle insulin resistance and adipose tissue hypertrophy persist beyond the reshaping of gut microbiota in young rats fed a fructose-rich diet. J Nutr Biochem. 2022 Dec 7;113:109247. doi: 10.1016/j.jnutbio.2022.109247. Epub ahead of print. PMID: 36496062.

12. Mills KL, Goddings AL, Clasen LS, Giedd JN, Blakemore SJ (2014) The developmental mismatch in structural brain maturation during adolescence. Dev Neurosci 36(3-4):147-60. https://doi.org/10.1159/000362328.

13. Trzeciak P, Herbet M. Role of the Intestinal Microbiome, Intestinal Barrier and Psychobiotics in Depression. Nutrients. 2021 Mar 12;13(3):927. doi: 10.3390/nu13030927. PMID: 33809367; PMCID: PMC8000572.

14. Spagnuolo, M.S.; Bergamo, P.; Crescenzo, R.; Iannotta, L.; Treppiccione, L.; Iossa, S.; Cigliano, L. Brain Nrf2 Pathway, Autophagy, and Synaptic Function Proteins Are Modulated by a Short-Term Fructose Feeding in Young and Adult Rats. Nutr. Neurosci. 2020, 23, 309–320.

15. Spagnuolo, M.S., Mazzoli, A., Nazzaro, M., Troise, A.D., Gatto, C., Tonini, C., Colardo, M., Segatto, M., Scaloni, A., Pallottini, V., Iossa, S., Cigliano, L. Long-Lasting Impact of Sugar Intake on Neurotrophins and Neurotransmitters from Adolescence to Young Adulthood in Rat Frontal Cortex. Mol Neurobiol. 2022. doi: 10.1007/s12035-022-03115-8.

16. Yamazaki M, Yamada H, Munetsuna E, Ishikawa H, Mizuno G, Mukuda T, Mouri A, Nabeshima T, Saito K, Suzuki K, Hashimoto S, Ohashi K. Excess maternal fructose consumption impairs hippocampal function in offspring via epigenetic modification of BDNF promoter. FASEB J. 2018 May;32(5):2549-2562. doi: 10.1096/fj.201700783RR. Epub 2018 Jan 8. PMID: 29401579.

Abbreviations

ACh acetylcholine

AChE acetylcholinesterase

AMP adenosine monophosphate

Atp5mpl mitochondrial membrane ATP synthase F(1)F(0)

Avp vasopressin-neurophysin 2-copeptin

BDNF brain-derived neurotrophic factor

pro-BDNF BDNF precursor

BSA bovine serum albumin fraction V

C control

Camk2b calcium/calmodulin-dependent protein kinase type II, subunit beta

CEL Nε-carboxyethyllysine

CI complex I

$CI_{\&IIE}$ the maximum capacity of the electron transport chain

$C_{I\&IIP}$ respiration supported by complexes I and II

C_{IIE} maximal capacity supported by CII alone d

CI_L leak respiration

CI_P phosphorylating respiration supported by complex I

CML Nε-carboxymethyllysine

Cox6b1 cytochrome c oxidase, subunit VIb polypeptide 1

CR control rescued

p-CREB phospho-cAMP response element- binding protein

CREB phospho-cAMP response element- binding protein

d4-lysine DL-lysine-4,4,5,5-d4 dihydrochloride

Erk1/2 extracellular signal-regulated kinase

Erlin2 ER lipid raft associated 2

F fructose-fed

FR fructose-rescued

FCCP carbonyl cyanide p-trifluoromethoxyphenylhydrazone

FCR flux control ratios

FCR_L leak respiration

FCR_P phosphorylating respiration

GABA γ-aminobutyric acid

GAR-HRP Goat anti-Rabbit-Horseradish peroxidase-conjugated IgGs

GAM-HRP Goat anti- Mouse-Horseradish peroxidase-conjugated IgGs

GFAP glial fibrillary acidic protein

Glut-4 glucose transporter-4

Glut-5 glucose transporter-5

GR glutathione reductase

GSSG glutathione

H2A histone family, member V

Hb hemoglobin

HD Huntington disease

HFCS high-fructose corn syrup

Hpt haptoglobin

HRR high resolution respirometry

Iba 1 ionized calcium binding adapter 1

IgG goat anti-rabbit horseradish peroxidase-conjugated

IL-1 interleukin-1

IL-6 interleukin-6

Ina internexin neuronal intermediate filament protein, alpha

LC–MS/MS liquid chromatography high resolution tandem mass spectrometry

LTD long-term depression

LTP long-term potentiation

MAO monoamine oxidase

MSI Metabolomics Standards Initiative

MW molecular weight

Ndufs1 NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial

Ndufb9 NADH dehydrogenase (ubiquinone) 1 beta subcomplex 9

NF-L neurofilament light

NF-M neurofilament-M

Nefm neurofilament medium polypeptide

NF-kB nuclear factor kappa-light-chain-enhancer of activated B cells

NGF nerve growth factor

N-Tyr Nitro-tyrosine

O2k Oxygraph-2k

OXPHOS mitochondrial complexes I-V

p75NTR tropomyosin receptor kinase B

Pcbd2 4a-hydroxytetrahydrobiopterin dehydratase

PINK-1 PTEN-induced putative kinase 1

PGC-1α peroxisome proliferatoractivated receptor gamma coactivator 1-alpha

PSD 95 post-synaptic density protein 95

PVDF polyvinylidene difluoride

ROS oxygen species

S100b protein S100-B

Slc6a7 solute carrier family 6 (neurotransmitter transporter, l-proline) member 7

SOD superoxide dismutase activity

Sorcs2 sortilin-related VPS10 domain containing receptor 2

TBARS thiobarbituric reactive substances

TEAB triethylammonium bicarbonate

TFAM mitochondrial transcription factor A

TH thyroxine hydroxylase

TMT Tandem Mass Tag

TrkA tropomyosin receptor kinase A

TrkB tropomyosin receptor kinase B

VDAC-1 voltage-dependent anion-selective channel 1

VDAC-2 voltage-dependent anion-selective channel protein 2

VDAC-3 voltage-dependent anion-selective channel protein 3

Appendix

Publications

1. Mazzoli A, Spagnuolo MS, Gatto C, **Nazzaro M**, Cancelliere R, Crescenzo R, Iossa S, Cigliano L. Adipose Tissue and Brain Metabolic Responses to Western Diet - Is There a Similarity between the Two? Int J Mol Sci. 2020 Jan 25;21(3):786. doi: 10.3390/ijms21030786

2. Tonini C, Segatto M, Martino F, Cigliano L, **Nazzaro M**, Barberio L, Mandalà M, Pallottini V. Effects of Late-Life Caloric Restriction on Age-Related Alterations in the Rat Cortex and Hippocampus. Nutrients. 2021 Jan 15;13(1):232. doi: 10.3390/nu13010232. PMID: 33467406; PMCID: PMC7830987

3. Mazzoli A, Spagnuolo MS, **Nazzaro M**, Gatto C, Iossa S, Cigliano L. Fructose Removal from the Diet Reverses Inflammation, Mitochondrial Dysfunction, and Oxidative Stress in Hippocampus. Antioxidants (Basel). 2021 Mar 20;10(3):487. doi: 10.3390/antiox10030487. PMID: 33804637; PMCID: PMC8003595.

4. Mazzoli A, Gatto C, Crescenzo R, Spagnuolo MS, **Nazzaro M**, Iossa S, Cigliano L. Gut and liver metabolic responses to dietary fructose - are they reversible or persistent after switching to a healthy diet? Food Funct. 2021 Aug 21;12(16):7557-7568. doi: 10.1039/d1fo00983d. Epub 2021 Jul 21. PMID: 34286786.

5. Spagnuolo MS, Mazzoli A, **Nazzaro M**, Troise AD, Gatto C, Tonini C, Colardo M, Segatto M, Scaloni A, Pallottini V, Iossa S, Cigliano L. Long-Lasting Impact of Sugar Intake on Neurotrophins and Neurotransmitters from Adolescence to Young Adulthood in Rat Frontal Cortex. Mol Neurobiol. 2022 Nov 17. doi: 10.1007/s12035-022-03115-8. Online ahead of print. PMID: 36394711

6. Mazzoli A, Di Porzio A, Gatto C, Crescenzo R, **Nazzaro M**, Spagnuolo M.S, Baccigalupi L, Ricca E, Amoresano A, Fontanarosa C, Bernacchioni C, Donati C, Iossa S, Cigliano L. Skeletal muscle insulin resistance and adipose tissue hypertrophy persist

beyond the reshaping of gut microbiota in young rats fed a fructose-rich diet. J Nutr Biochem. 2022 Dec 7;113:109247. doi: 10.1016/j.jnutbio.2022.109247.

7. D'Ambrosio C, Cigliano L, Mazzoli A, Matuozzo M, **Nazzaro M**, Scaloni A, Iossa S, Spagnuolo MS. Fructose Diet-Associated Molecular Alterations in Hypothalamus of Adolescent Rats: A Proteomic Approach. Nutrients. 2023 Jan 16;15(2):475. doi: 10.3390/nu15020475. PMID: 36678346

List of communication

1. L. Cigliano, A. Mazzoli, **M. Nazzaro**, M. S. Spagnuolo, C. Gatto, D. C. Maresca, S. Iossa. Impact of dietary fructose on brain mitochondria and oxidative stress. 23rd International Conference on Oxidative Stress Reduction of the International Society of Antioxidants, Redox Homeostasis and Antioxidants, Paris October 13-15 (2021).
2. A. Mazzoli, M.S. Spagnuolo, **M. Nazzaro**, C. Gatto, S. Borrelli, I. D'Angelo, S. Iossa, Luisa Cigliano. Effect of short-term fructose-rich diet on brain: what is reversible andcwhat persists after sugar removal from the diet? 71th National Congress Italian Physiological Society (SIF), 7-9 September (2021).
3. Crescenzo R, Spagnuolo MS, Iannotta L, Cancelliere R, Mazzoli A, Gatto C, Canè M, N**azzaro M**, Iossa S, Cigliano L. Initial Brain Aging and High Fat-High Fructose Diet: Effect on Mitochondrial Bioenergetics, Oxidative Status and Cholesterol Homeostasis in Rat Brain. Federation of European Physiological Societies (FEPS) and the Italian Physiological Society (SIF), Bologna, 10-13 Settembre (2019).
4. M.S. Spagnuolo, L. Iannotta, **M. Nazzaro**, M Canè, R. Crescenzo, S. Iossa, L. Cigliano. Short term fructose-rich diet affects markers of neuronal survival and differentiation in young and adult rats. 68th National Congress Italian Physiological Society (SIF) Pavia: 22, 180 (2017).