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THE NEURODEGENERATION PROCESS IN THE STRIATUM OF MICE EXPOSED TO OBESOGENIC DIETS

Ankita Talukdar

Master's Degree Project in Molecular Biology, 60 cr
2020

Department of Biology
Lund University

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THE NEURODEGENERATION PROCESS IN THE STRIATUM OF MICE EXPOSED TO OBESOGENIC DIETS

Master Degree Thesis

Ankita Talukdar

Supervisor
João Duarte
Principal investigator at Diabetes and Brain function lab
Department of Experimental Medicine
Lund University

Master's Degree Project in Molecular Biology, general programme [2018-2020]

Department of Biology
Lund University

TABLE OF CONTENTS

Abbreviations

6-OHDA- 6-hydroxydopamine
AD- Alzheimer's disease
BDNF- Brain-derived neurotrophic factor
CD – Control diet
DA- Dopamine
GABA- γ-Aminobutyric acid
GFAP- Glial fibrillary acidic protein
HFD- High Fat Diet
HFHSD- High Fat, High Sucrose Diet
Iba1- Ionized calcium binding adaptor molecule 1
IR- Insulin receptor
IRS- Insulin receptor substrate
MPTP- 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN- Medium Spiny Neurons
PBS- Phosphate buffered saline
PD- Parkinson's disease
SNAP25- Synaptosome associated protein 25kDa
SNARE- Soluble N-ethylmaleimide sensitive factor Attachment protein Receptor
T2D- Type 2 Diabetes
TH- Tyrosine Hydroxylase
VGLUT 1& 2- Vesicular glutamate transporters 1 & 2

Popularised summary

THE NEURODEGENERATION PROCESS IN THE STRIATUM OF MICE EXPOSED TO OBESOGENIC DIETS

Master's Degree project in Molecular Biology, 60 credits

Ankita Talukdar

Supervisor: *Joao Duarte*

Diabetes and Brain function lab,
Department of Experimental Medicine

Project runs: *2019-09-02 – 2020-06-05*

Project description:

The striatum is a nucleus (cluster of neurons) in the forebrain that is involved in reward behaviour, motor functions, inhibition and impulse control.

The consumption of diets rich in sugar and/or saturated fats induces obesity and overall affects metabolism. Such diet-induced metabolic changes progress to the development of type 2 diabetes (T2D), which impairs normal brain function. In particular, changes in neurons of the striatum are likely to cause perturbations on emotional behaviour.

In this study, neuronal damage in striatum was investigated in mice consuming high-fat diets by analysing levels of proteins involved in synaptic function, as well as analysing microglia and astrocytes, which are key brain cells in the maintenance of neuronal integrity.

Our results demonstrate that high-fat diets cause some degree of synaptic damage, depicted by a reduction of synaptic protein levels, without substantial changes in the phenotype of microglia and astrocytes. The impact of these changes in the function of the striatum remain to be studied.

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Abstract

Obesity and diabetes are known to impact brain function and alter metabolism. Studies in rodents show that consumption of high fat and high sugar diets lead to brain dysfunction affecting memory and cognition, and increases risk of cerebro-vascular and neurodegenerative disorders. Striatum, which is involved in reward-based behaviour and motor control, undergoes changes on exposure to such diets leading to movement defects, loss of impulse control, increased susceptibility to neurotoxins like MPTP and 6-OHDA. This effect is brought about by insulin resistance and loss of dopaminergic neurons thereby reducing dopamine signalling. In this study, striatal degeneration was investigated by measuring levels of synaptic proteins by immunoblotting and evaluating the phenotype of glial cells in mice exposed to fat-rich diets (10%, 45% and 60% of total energy intake) for 6 months. Relative to control fed the 10%-fat diet, mice fed 60% fat showed a decrease in levels of synaptic proteins Synaptosome-associated protein (SNAP25) and Syntaxin-1 whereas 45% fat diet reduced levels of Synaptophysin. The data from this study does not point to significant changes in dopamine signalling, astrogliosis or neuroinflammation. In sum, the trend observed in terms of reduction in levels of synaptic proteins that are crucial for maintaining efficient synaptic plasticity is likely to cause to neuronal networks involving the striatum, which controls, motor function and emotional behaviours.

Introduction

Obesity arises due to the imbalance in energy intake and expenditure which in turn increases the risk of developing Type 2 Diabetes (T2D). Insulin resistance, a characteristic trademark of obesity and T2D, impacts peripheral tissue as well as brain function. Clinical complications of obesity include increased inflammation, increased risk of vascular disease, movement disorders and deficits in learning, memory (Pistell et al., 2010; Kleinridders et al., 2019).

Food consumption is regulated by the homeostatic system of hypothalamus and the reward system of mesolimbic dopaminergic pathways. Food intake controlled by homeostatic system through hypothalamic neurons is regulated by satiety signals and hormones such as insulin and leptin whereas physiological stimuli like taste, hunger, palatable food intake controls the reward system. The deregulation of these systems is linked to obesity further promoting a preference for high calorie diet, which results in a cycle of over-eating causing long-term obesity and development of T2D. In addition, these systems also play a role in emotional behaviour and their deregulation results in mood disorders such as anxiety and depression (Lutter et al., 2009; Kleinridders et al., 2019).

A number of studies have investigated the link between T2D and neurodegenerative disorders. Reduction in insulin signalling plays a role in the

pathogenesis of Alzheimer's disease (AD) along with factors such as oxidative damage. A study conducted on post-mortem AD patient brains aged 62-94 years at death showing oxidative stress in caudate of striatum illustrates that nucleic acid damage plays a role in age-associated neurodegeneration (Kleinridders et al., 2014; Li et al., 2020). Deterioration of dopaminergic (DA) neurons from midbrain paves the way for Parkinson's disease (PD) pathogenesis resulting in loss of motor functions and reduction in dopamine signalling (Olanow et al., 2009). This loss of DA neurons is brought about by a reduction of Insulin receptor (IR) mRNA and increased levels of Insulin receptor substrate (IRS) phosphorylation with inhibitory action on insulin signalling leading to enhanced insulin resistance (Duarte et al., 2012; Morsi et al., 2018; Fiory et al., 2019).

The striatum or corpus striatum is a cluster of neurons in the forebrain that receives glutamatergic inputs from the cortex and dopaminergic inputs from the substantia nigra and ventral tegmental area, and it serves as the primary input source for basal ganglia. It plays an important role in the motor functions like action planning, decision making and reward-based behaviour like motivation, reinforcement and salience (Báez-Mendoza and Schultz, 2013).

Functionally, the striatum is divided into a ventral and dorsal side. The ventral striatum consisting of nucleus accumbens (NAc) and olfactory tubercle plays an important role in reward-based learning. The dorsal striatum consists of caudate nucleus and putamen and is primarily involved in motor functions and inhibitory and impulsivity control. The dominant neurons in the striatum are GABAergic medium spiny neurons (MSN) of two subtypes: D1-MSN (direct pathway; excitatory functions) and D2-MSN (indirect pathway; inhibitory functions) (Báez-Mendoza and Schultz, 2013).

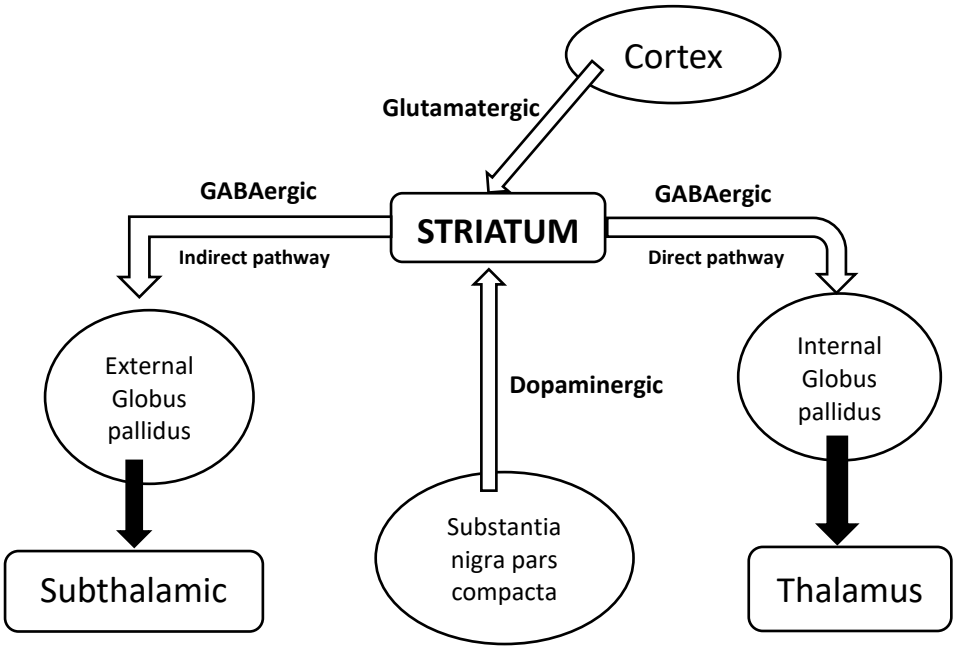

Figure 1: Schematic diagram showing the input and output targets of striatum.

The rewarding value of food consumption is encoded by DA signalling in striatum. Upon consumption of palatable food and certain drugs, DA releasing neurons are activated and project from ventral tegmental area to ventral striatum (South et al., 2008). The synaptic levels of dopamine are regulated by DA transporters which reduce under the effect of high calorie diets resulting in an imbalance in DA levels. This imbalance results in reduction of impulse control leading to altered food seeking and binge-like eating behaviour (Speed et al., 2011; Adams et al., 2015; Fritz et al., 2018). Along with altered behaviour, DA signalling was studied to be involved in motor disorders. In the study by Jang et al., high fat-fed mice displayed decreased movement in open field tests and increased missteps in vertical grid tests which was linked to reduction of Tyrosine hydroxylase (rate-limiting enzyme for dopamine production) in striatum and substantia nigra (Jang et al., 2017). Contrary to these results, DA signalling under high fat diet did not affect learning and performance in striatal-dependent memory tasks despite reduction in DA levels in rats (Nguyen et al., 2017).

Mechanisms by which high fat and/or sugar diets impact striatal function are not fully understood. In this study we tested the hypothesis that synaptic degeneration is involved in the development of obesity-induced striatum damage. Namely, we investigated the degree of damage caused by several high-fat consumption levels by analysing levels of synaptic proteins that are involved in neurotransmitter release and regulation of synaptic plasticity, alterations in striatal dopamine homeostasis, and phenotype of astrocytes and microglia.

Materials and Methods

- Animals and diets

HFD feeding is used extensively as a model to understand the underlying causes for obesity and its associated metabolic alterations. Diets rich in saturated fatty acids were found to be more damaging on metabolic profile than unsaturated fats. Mice model was exposed to HFD feeding with varying percentage of lard-based fat which is rich in saturated fats which were reported to show a phenotype of obesity with higher body weight compared to mice fed CD. Alterations in fasting blood glucose and plasma insulin levels indicated perturbations in glucose homeostasis observed within few weeks of HFD feeding and HFD60 group shows alterations earlier than those in HFD45. Circulating levels of leptin was also higher in HFD mice when compared to CD mice. Reversibility of diet (HFD60 to CD) shows a restoration in glucose homeostasis, decrease in body weight and normalization of metabolic biomarkers (leptin and plasma insulin levels). Markers indicating liver damage such as Aspartate aminotransferase (AST) and Alanine transaminase (ALT) increased under HFD and remains unchanged with reversal of diet suggesting presence of liver damage. These alterations indicate

that the amount of fat in diet influences metabolic deregulation (Soares, Duarte et al. 2018)

All procedures on animals were approved by the Malmö/Lund Committee for Animal Experiment Ethics (permit number 994/2018), and are reported following the ARRIVE guidelines (Animal Research: Reporting *in vivo* Experiments, NC3Rs initiative, UK). Male C57BL/6J mice were obtained from Taconic (Ry, Denmark) at 8 weeks of age and allowed to acclimatise to the animal facility for one week. Mice were housed in groups of 4-5 on a 12-hour light-dark cycle with lights on at 07:00, room temperature at 21-23 °C, humidity at 55-60%, and access to tap water and food *ad libitum*.

Control mice were fed a low-fat diet with 10% kcal from fat, 20% kcal from protein and 70% kcal from carbohydrates (D12450J, Research Diets, New Brunswick, NJ, USA). High fat, high sucrose diet (HFHSD) exposed mice were fed for 30 days a diet with 60% kcal from fat, 20% kcal from protein and 20% kcal from carbohydrates (D12492, Research Diets) and were also supplemented with a 20% (w/v) sucrose solution in addition to tap water. Mice were also fed standardised high fat diets (HFD) containing 10% (CD), 45% (HFD45) and 60% (HFD60) kilocalories from lard-based fat (D12450B, D12451, D12492, Research Diets, New Brunswick, NJ, United States) for a period of 6 months. A reversed diet group (HFD-Rev) was fed a high fat diet (60% kilocalories) for 4 months and switched to CD for the last 2 months in the study.

At the end of the diet period, mice were briefly anaesthetised with isoflurane and decapitated. Striatum tissue was collected and stored at -80°C for further experiments.

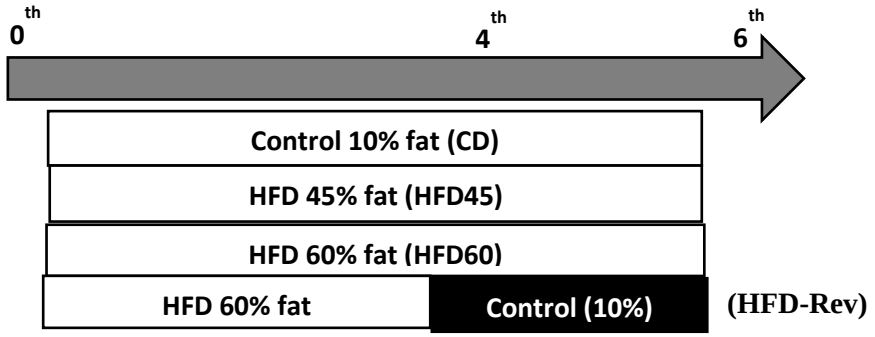

Figure 2: Experimental design for mice fed high fat diet for 6 months. Mice were randomly selected for four diet groups- CD (10% kcal from fat, n=6), HFD45 (45% kcal from fat, n=6), HFD60 (60% kcal from fat, n=6) and HFD-Rev (60% kcal fat for 4 months followed by 10% kcal from fat for 2 months, n=5)

- Sample preparation

Striatum tissue stored at -80°C was thawed and lysed using lysis buffer with protease inhibitors (in mmol/L: 150 NaCl, 1 ethylenediaminetetraacetic acid (EDTA), 50 tris(hydroxymethyl)aminomethane (Tris)-HCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) sodium dodecylsulfate (SDS), pH 8.0). Samples were homogenised using ultrasonic homogenizer. Centrifugation (3000g, 10 minutes, 4°C) was performed to collect the supernatant. Protein quantification was done using bicinchonic acid assay (kit from Pierce, Thermo Fisher Scientific) and supernatant stored at -20°C for Western blotting. (n=6 for Control and HFD45, HFD60; n=5 for HFD-Rev; n=3 for Control and HFHSD)

- Measures of protein expression by western blotting

Samples were denatured in SDS buffer (100uL reducing agent + 900uL SDS, Invitrogen, ThermoFisherScientific) and equal amounts of protein (5-15ug/lane) were electrophoretically separated in polyacrylamide gels (NuPAGE 4-12% bis-tris gel, Invitrogen, ThermoFisherScientific) along with molecular weight standards (Precision Plus Protein standards, Bio-Rad) in 1x MOPS (3-(N-morpholino)propane sulfonic acid) running buffer 125V for 75 minutes. They were then blotted onto nitrocellulose membranes (GE Life Sciences, Sweden) in blotting buffer (192 mmol/L glycine, 25 mmol/L Tris and 20% (v/v) methanol, pH 9.2) 110V for 90 minutes and blocked in 5% milk and/or 5% BSA in TBS-T (Tris-buffered saline (TBS) (in mmol/L: 20 Tris, 137 NaCl, pH 7.46) with 0.1% (v/v) tween-20) for 1.5h at RT. Blots were analysed using primary antibodies (Table 1). After incubation with primary antibodies (overnight, 4°C), blots were washed using TBS-t and incubated with HRP-conjugated secondary antibodies (1-2h, RT) (Table 2). The blots were visualized using enhanced chemiluminescent substrate (Super Signal West Pico Plus, Thermofisher) and images obtained using a ChemiDoc+ (Bio-Rad). Blot images were scanned and analysed using Image Lab software (Bio-Rad).

Table 1: List of Primary antibodies used in the present study.

	Primary antibody	**Host**	**Dilution**	**Manufacturer**	**Molecular weight (kDa)**
1	anti-PSD95	Rabbit	1:2000	Abcam, ab76115	95
2	anti-Gephyrin	Rabbit	1:1000	Abcam, ab181382	~93
3	anti-Dopamine transporter-1	Mouse	1:2000	Novus, NBP2-22164SS	65-70
4	anti-Tyrosine hydroxylase	Rabbit	1:1000	Novus, NB300-109	55-58
5	anti-GFAP	Rabbit	1:1000	Sigma, HPA056030	50
6	anti-VGLUT1	Mouse	1µg/mL	Novus, NBP2-46627	~61

7	anti-VGLUT2	Rabbit	1:1000	Abcam, ab216463	55-60
8	anti-Syntaxin-1	Rabbit	1:1000	Millipore	34
9	anti-Syntaxin-4	Rabbit	1:1000	Abcam, ab184545	34
10	anti-Synaptophysin	Rabbit	1:10000	Abcam, ab32127	37
11	anti-SNAP25	Rabbit	1:5000	Abcam, ab109105	25
12	anti-Iba1	Rabbit	1:1000	Wako, 016-200001	17

Table 2: List of Secondary antibodies used in the present study.

	Secondary antibody	**Dilution**	**Manufacturer**
1	Goat anti-Rabbit	1:10000	Abcam (ab6721)
2	Goat anti-Mouse	1:10000	Abcam (ab6789)

- Immunostaining for Microglia and GFAP+ cells

Microglia cells and astrocyte were fluorescent immunolabelled using microglia marker Iba1 and astrocyte marker GFAP. Brains were briefly fixed in phosphate buffered formaldehyde (Histolab, Askim, Sweden) and stored in a 30% sucrose solution in PBS at 4°C. Immunostaining was carried out in 20µm cryostat-sectioned coronal brain slices. Blocking solution containing 5% goat serum, 0.5% Triton X-100 and 1% BSA in 1X PBS was used to incubate slices (1h, RT). Primary antibodies Anti-Iba1(Wako; rabbit; 1:200), Anti-GFAP tagged AF-488 (Thermofischer; rabbit; 1:500) and secondary antibody AF-568 (Thermofischer; rabbit; 1:500) were used for incubating slices (overnight, 4°C; 1.5h, RT) respectively. The slices were washed using 1X PBS for 45 minutes after each incubation step and mounted with DapiMount anti-fade mounting media (Invitrogen). Slides were examined under a Nikon A1RHD confocal microscope with 20x Plan Apo objective, NA 0.75 (Nikon instruments, Tokyo, Japan) and images were acquired with NIS-elements, version-5.20.01 (Laboratory Imaging, Nikon). Images were processed using ImageJ (NIH, Bethesda, MD, USA).

- Data analysis

Western blots were analysed using Image lab software. The blots were scanned for lane and band detection and analysed densitometrically for quantification of protein expression. Data were calculated as ratio of expression over average of control sample expression. Protein expression in Control and HFD or HFHSD were calculated and presented as total reactivity (relative to control) within groups.

For the evaluation of effects of diet on glial cells, n=6 from Control and HFHSD animals (n= 3 for Iba1 and n=3 for GFAP) were selected and analysed using ImageJ software. Images were adjusted to a suitable threshold intensity (10%-15%) and analysed for area and perimeter. Cell counting was done manually for microglia cells. Also, morphology of microglia cells was analysed depending on shape and length of processes.

- Statistics

Data was analysed using GraphPad Prism 8.4.2 software (GraphPad Software, Inc., San Diego, California, USA). The results for the effect of HFD on mice striatum was analysed using analysis of variance (ANOVA) followed by Fischer's least significant difference test (LSD) for independent comparisons. The results for HFHSD was analysed using two-tailed t-test. Two-way ANOVA was used for analysing the different types of microglia cells. All data are represented as mean ± SD. Statistical significance was accepted at $p<0.05$.

Results

- Metabolic syndrome phenotype in mice exposed to HFD and HFHSD

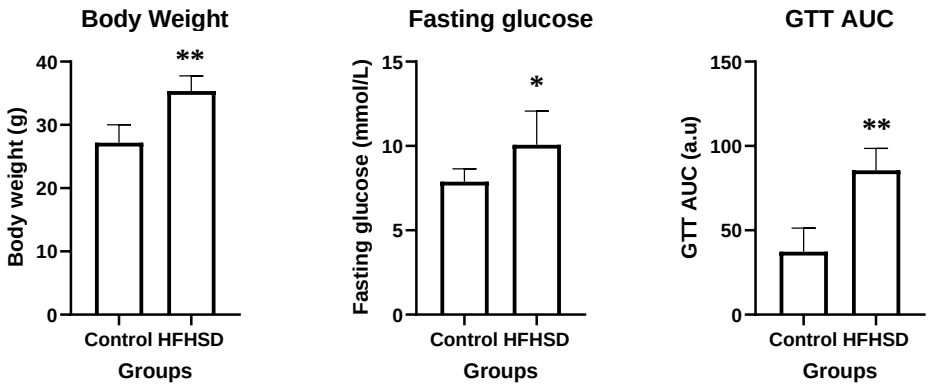

Figure 3: Mice used for the study showing body weight for HFHSD compared to CD. Glucose levels (mmol/L) measured after 6h fasting period and after administration of glucose i.p. (2g/kg). Data for area under the curve for oral glucose tolerance test after 6h fasting period expressed in mmol.h/L. n=6 for CD and HFHSD. Data represented as Mean ± SD. Symbols indicate significant difference in Unpaired t-tests (Mann-Whitney test). * and ** represent $p<0.05$ and $p<0.01$ respectively.

The HFD animals used in this study were reported in a previous study displaying obese phenotype (Soares, Duarte et al. 2018). Compared to controls, HFD-exposed mice for 6 months had increased weight grain, increased fasting blood glucose, increased insulinaemia, and decreased glucose tolerance, which are all proportional to the amount of fat in the diet (45% or 60%), and recover upon diet reversal to 10%-fat diet. Similarly, mice exposed to HFHSD for 1 month display

increased weight gain, increased fasting glycemia, and reduced glucose tolerance (all $p<0.05$; n=6).

- Effects of HFD and HFHSD on synaptic protein levels

In order to investigate the effects of diet on striatal degeneration, levels of synaptic proteins were measured. The levels of SNAP25 decreased with the increase in fat content and recovered in the reverse group, with protein levels significantly decreasing in HFD60 compared to control ($p=0.020$). Synaptophysin levels significantly reduced for HFD45 compared to control ($p=0.010$) but did not show any reduction in HFD60. Synaptic proteins such as Syntaxin-4 (S4) and Syntaxin-1 (S1) involved primarily in exocytosis in nerve terminals were analysed. S4 showed no significant impact of high fat on their levels ($p=0.827$) whereas levels of S1 significantly decreased for HFD60 ($p=0.025$) and HFD-Rev ($p=0.001$) compared to control and HFD-Rev ($p=0.043$) compared to HFD45.

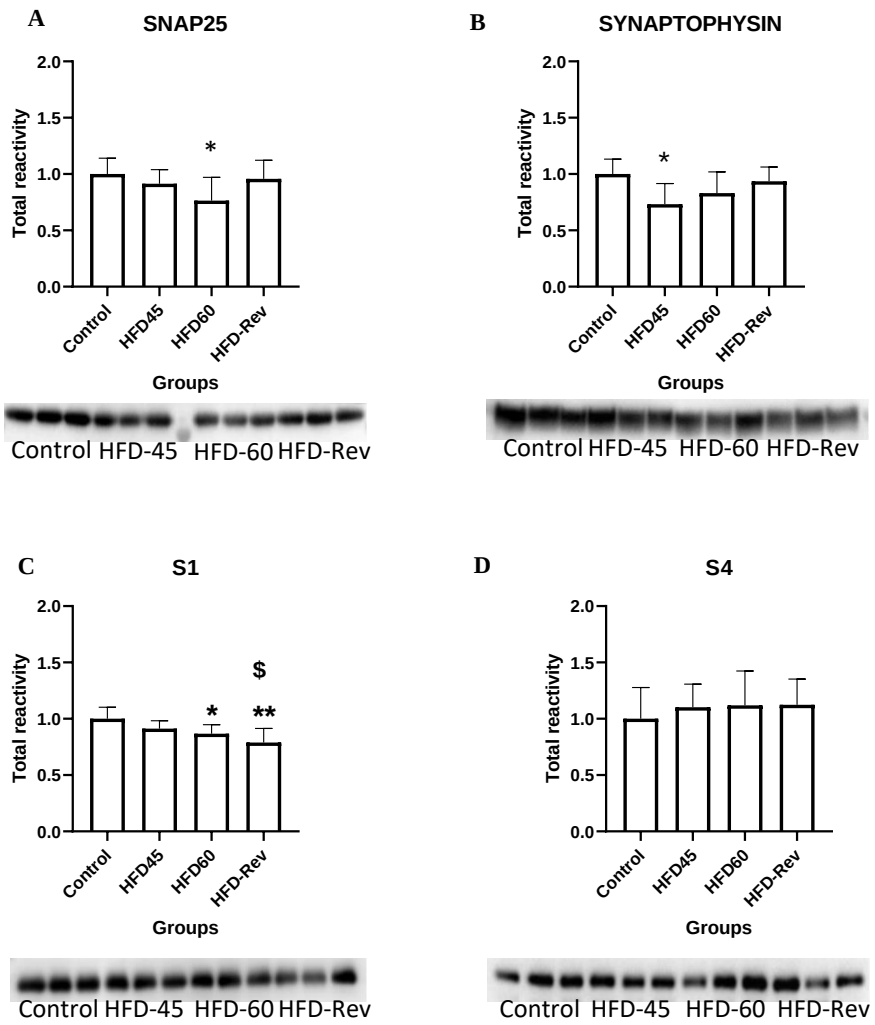

Figure 4: HFD induced changes in levels of Pre-synaptic proteins. Western blot results for SNAP25 (A), Synaptophysin (B), Syntaxin1 (C) and Syntaxin4 (D). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. Symbols indicate significant differences in LSD after ANOVA; *, ** represent $p<0.05$ and $p<0.01$ respectively when comparing diet groups with control whereas $ represent $p<0.05$ when comparing HFD45 to HFD-Rev. n=6 for Control, HFD45 and HFD60, n=5 for HFD-Rev.

Proteins present in the post-synaptic region of synapses were also analysed to investigate the effect of obesogenic diet on striatum. Postsynaptic density protein 95 (PSD95) shows no change across groups except significant decrease in the HFD-Rev group compared to control (p=0.047). Levels of gephyrin, a protein present in the post-synaptic zone of inhibitory synapses, changes significantly for HFD-Rev when compared to control (p=0.015) and HFD45 (p=0.018).

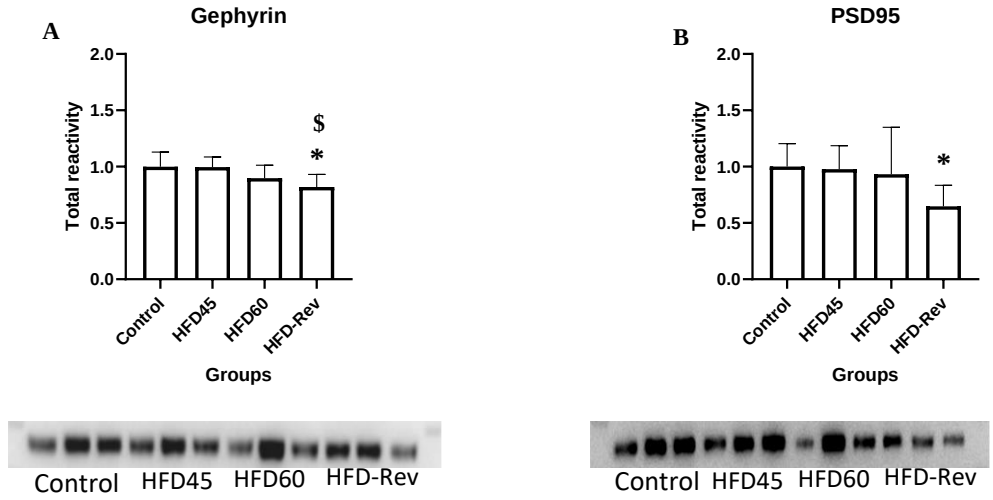

Figure 5: HFD induced changes in levels of Post-synaptic proteins. Western blot results for Gephyrin (A) and PSD95 (B). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. Symbols indicate significant differences in LSD after ANOVA; * represent $p<0.05$ when comparing diet groups with control whereas $ represent $p<0.05$ when comparing HFD45 to HFD-Rev. n=6 for Control, HFD45 and HFD60, n=5 for HFD-Rev.

The levels of vesicular glutamate transporters were analysed. VGLUT1 showed no changes in their expression across the groups. VGLUT2 level decreased in HFD45 compared to control but does not change for the other groups.

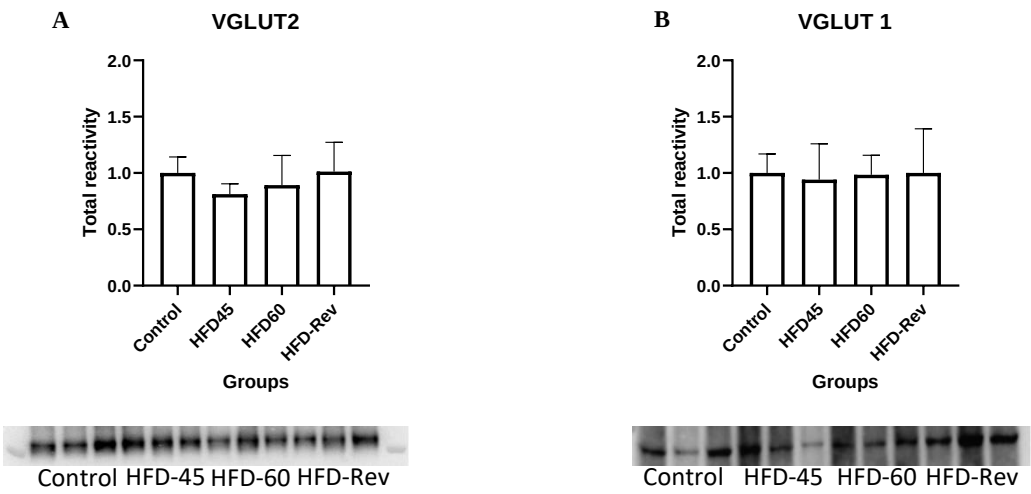

Figure 6: HFD induced changes in levels of Transporter proteins. Western blot results for VGLUT2(A) and VGLUT1(B). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. n=6 for Control, HFD45 and HFD60, n=5 for HFD-Rev.

Furthermore, synaptic protein levels were also examined for mice under HFHSD. Levels of SNAP25, Synaptophysin and Syntaxin-1/4 did not change significantly in HFHSD mice when compared to controls. Nevertheless, there was a general tendency for reduction of SNAP25 and Syntaxin-1/4 (Figure 7).

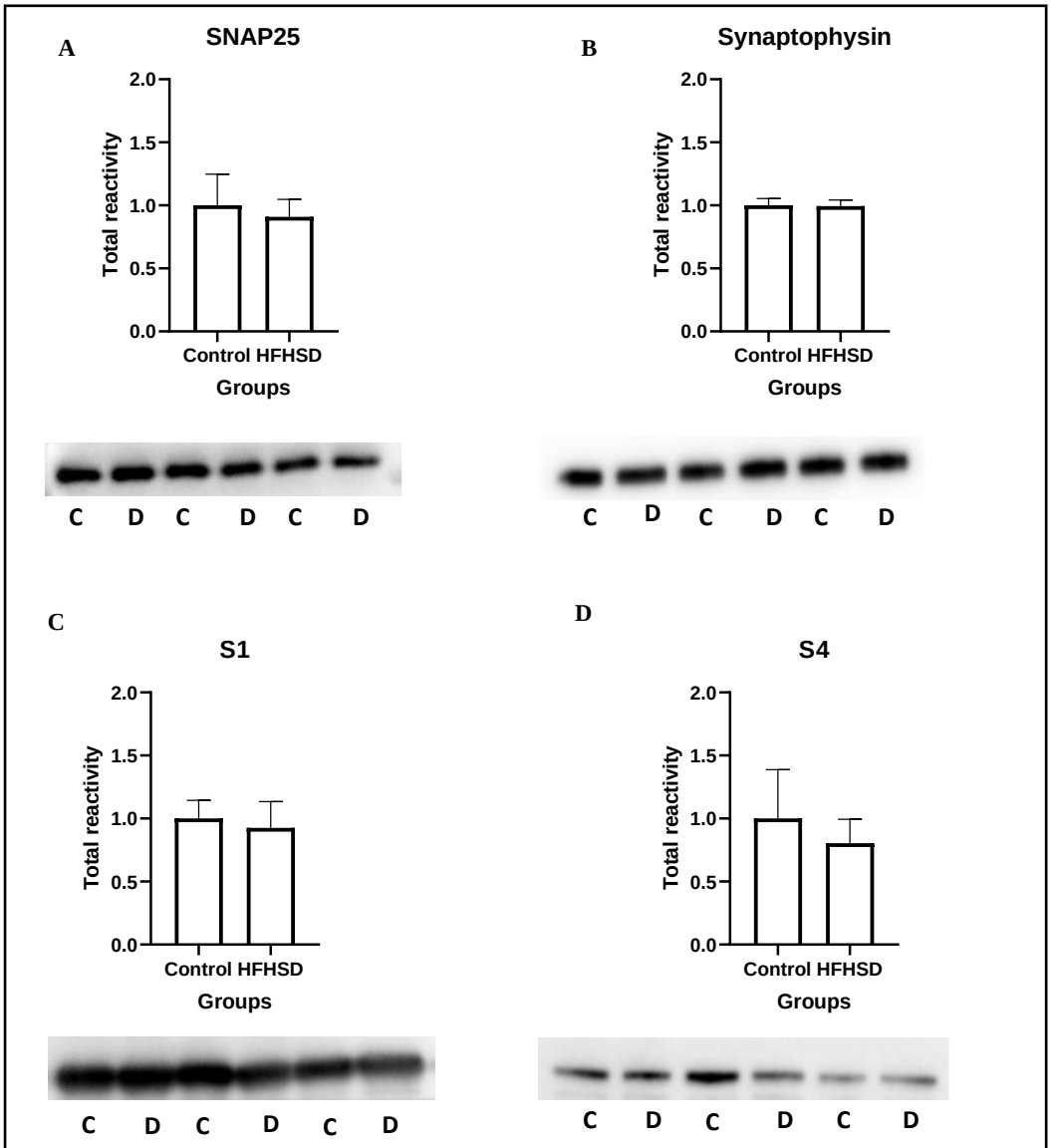

Figure 7: HFHSD induced changes in levels of Pre-synaptic proteins. Western blot results for SNAP25(A), Synaptophysin (B), Synatxin1 (C) and Syntaxin4 (D). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. n=3 for each group.

Levels of the vesicular transporter VGLUT2 was increased in the striatum of HFHSD-fed mice compared to controls (Figure 8A).

PSD95 levels shows an increase under HFHSD diet (p=0.057), while gephyrin was not modified (Figure 8B and 8C).

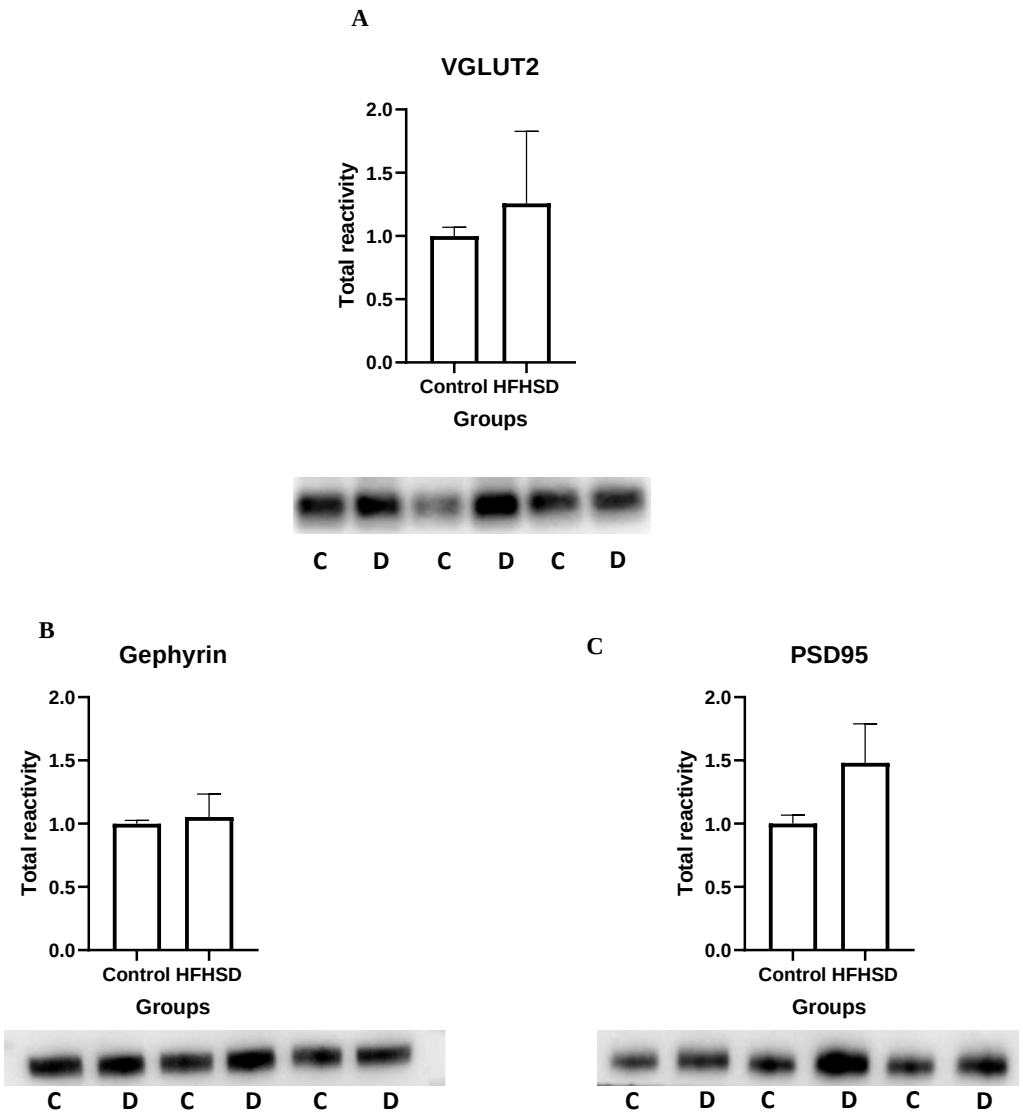

Figure 8: HFHSD induced changes in levels of Synaptic proteins. Western blot results for VGLUT2(A), Gephyrin (B) and PSD95 (C). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. n=3 for each group.

- Dopamine homeostasis

Dopamine transporter 1 (DAT1) is an essential protein involved in the reuptake of dopamine from synapses. DAT1 levels show a decreasing trend in HFD60 compared to control and a minor recovery in HFD-Rev (Figure 9A). However, no significant changes in DAT1 levels were observed across the diet groups. Similarly, DAT1 levels for mice under HFHSD show a similar trend where decrease of activity is observed when compared to control (Figure 10A).
Tyrosine hydroxylase (TH) is a crucial enzyme that converts tyrosine to L-Dopa, a precursor of neurotransmitter dopamine. TH activity increases for HFD45 and

HFD60 groups but no significant changes were observed in relation to control (Figure 9B). TH for HFHSD showed no changes in their activity when compared to control (Figure 10B).

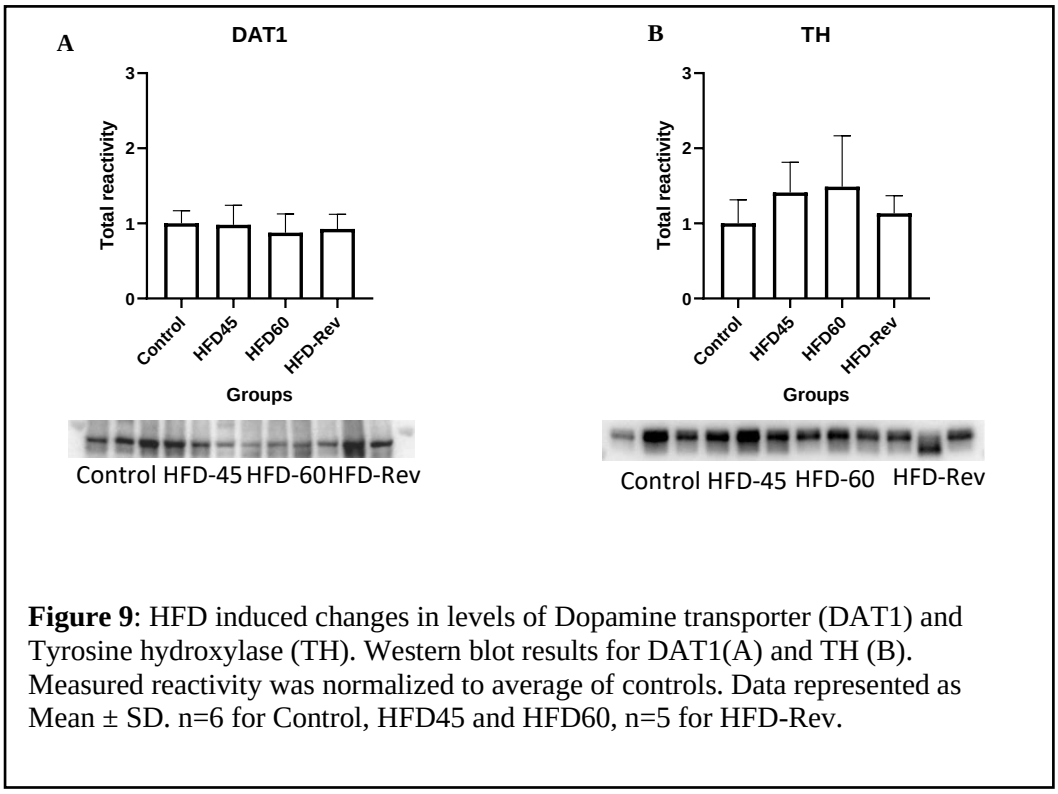

Figure 9: HFD induced changes in levels of Dopamine transporter (DAT1) and Tyrosine hydroxylase (TH). Western blot results for DAT1(A) and TH (B). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. n=6 for Control, HFD45 and HFD60, n=5 for HFD-Rev.

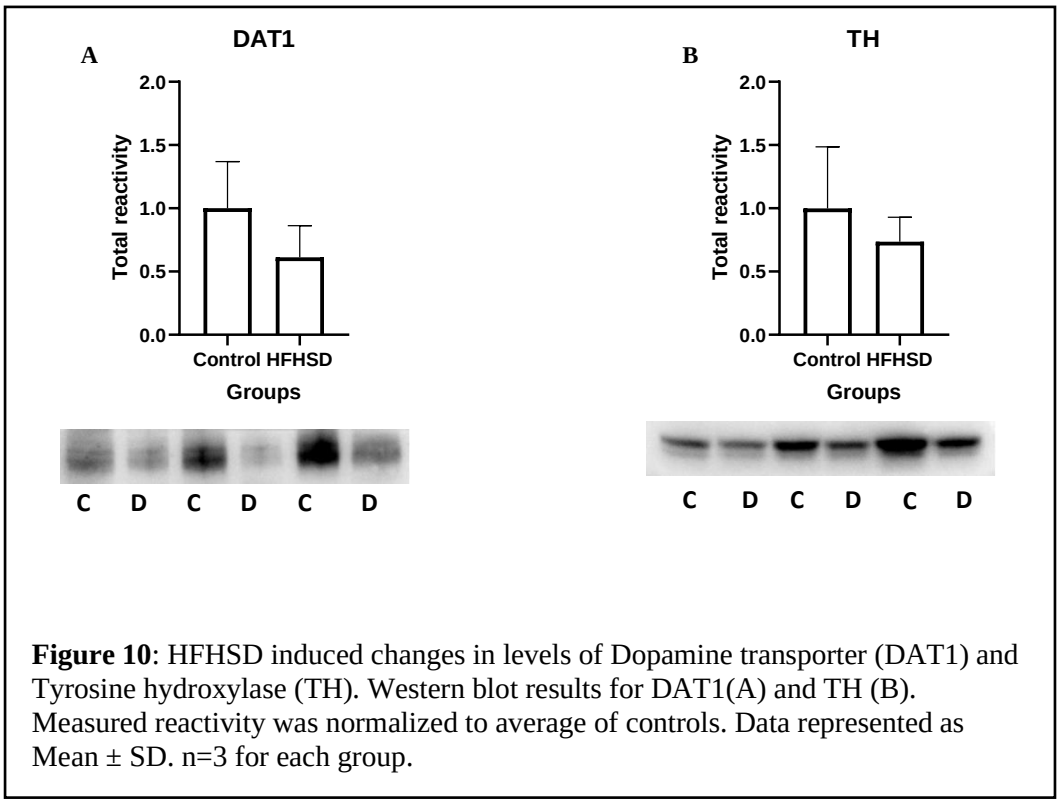

Figure 10: HFHSD induced changes in levels of Dopamine transporter (DAT1) and Tyrosine hydroxylase (TH). Western blot results for DAT1(A) and TH (B). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. n=3 for each group.

- Glial cell markers

Glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed in astrocytes, is considered a marker for reactive astrogliosis. In turn, the ionised calcium-binding adaptor molecule 1 (Iba1) is expressed in microglia and macrophages and its levels are often increased upon neuroinflammation. GFAP shows no changes across the groups for animals fed HFD (Figure 11A) or HFHSD (Figure 12A). Iba1 tends to increase in HFD-fed mice (Figure 11B) and it significantly decreases in the reverse group when compared to HFD60 ($p = 0.033$). Iba1 levels did not change in HFHSD (Figure 12B).

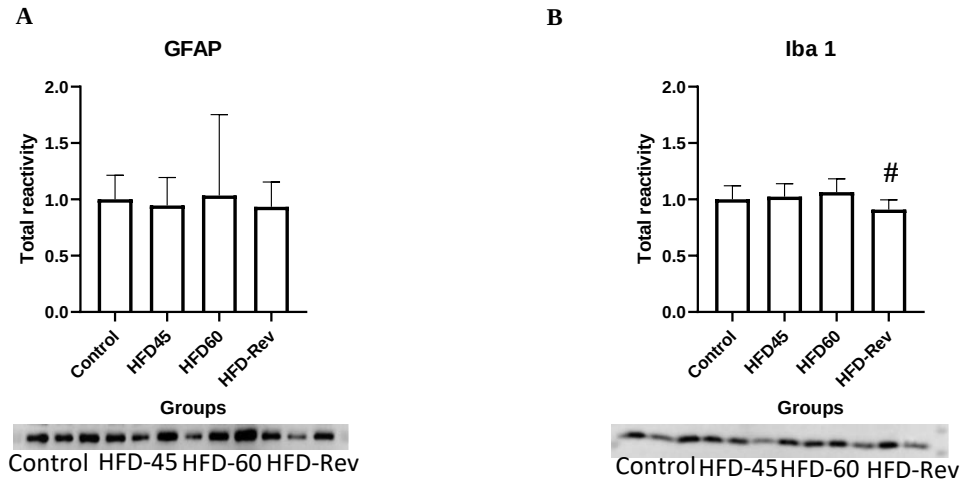

Figure 11: HFD induced changes in levels of Glial cell markers. Western blot results for GFAP(A) and Iba1 (B). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. Statistical significance accepted at $p<0.05$ after ANOVA; # represents $p<0.05$ when comparing HFD60 to HFD-Rev. n=6 for Control, HFD45 and HFD60, n=5 for HFD-Rev.

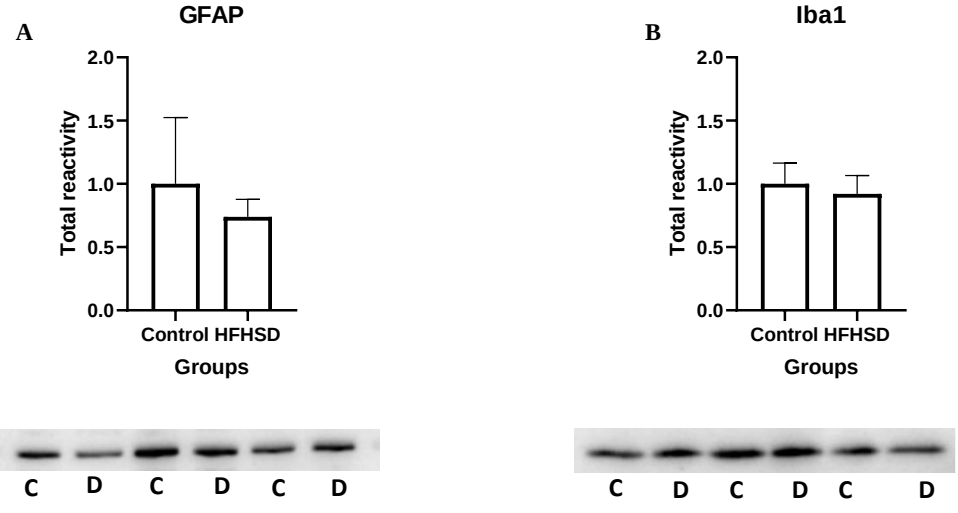

Figure 12: HFHSD induced changes in levels of Glial cell markers. Western blot results for GFAP(A) and Iba1 (B). Measured reactivity was normalized to average of controls. Data represented as Mean ± SD. n=3 for each group.

- Microglia and GFAP+ cell measures from staining

Activation of glial cells in striatum of mice fed with HFHSD was investigated by protein markers specific to microglia and astrocytes via immunofluorescence. The area and number of microglia, that is iba1-positive cells, were both slightly higher in HFHSD group compared to controls (Figure 13). However, no significant changes were observed for either parameter. Microglia morphology was analysed where cells were grouped based on their level of activation (Figure 13C). Resting microglia show thin branching processes that extend outward from a small cell body (ramified morphology) whereas activated microglia (hyper-ramified, bushy and amoeboid morphology) undergo marked changes like enlarged cell body, thicker and shorter processes, more branching of processes, transition from oblong to round shape (Raineri et al., 2012). In this study, the amount of amoeboid and ramified cells was not impacted by HFHSD but there was a tendency for increased number of bushy ($p=0.056$) and hyper-ramified cells ($p=0.074$) in HFHSD-fed mice relative to controls (Figure 13C).

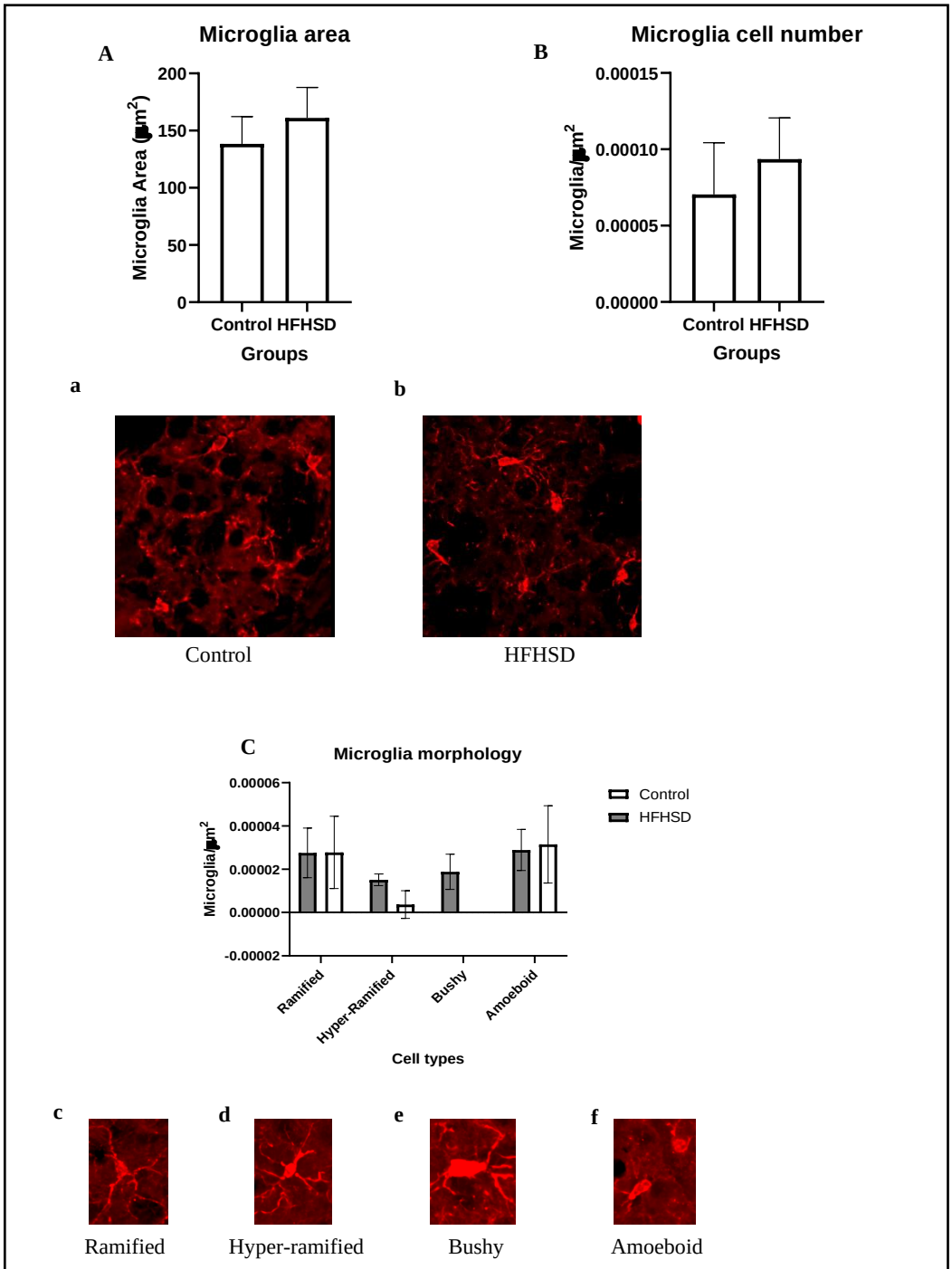

Figure 13: Immunofluorescence images of Control (a) and HFHSD (b) animal showing microglial cells expressing Iba1 (Red). Area occupied by cells (A) and number of cells per square micrometre of area (B) within striatum of mice. Microglia morphology analysed based of shape and number of processes (c-f). n=3 animals per group.

GFAP+ astrocytes were measured to as indication of reactive astrogliosis. The area of GFAP+ cells and the number of cells in diet group did not change significantly when compared to control (Figure 14 A & B).

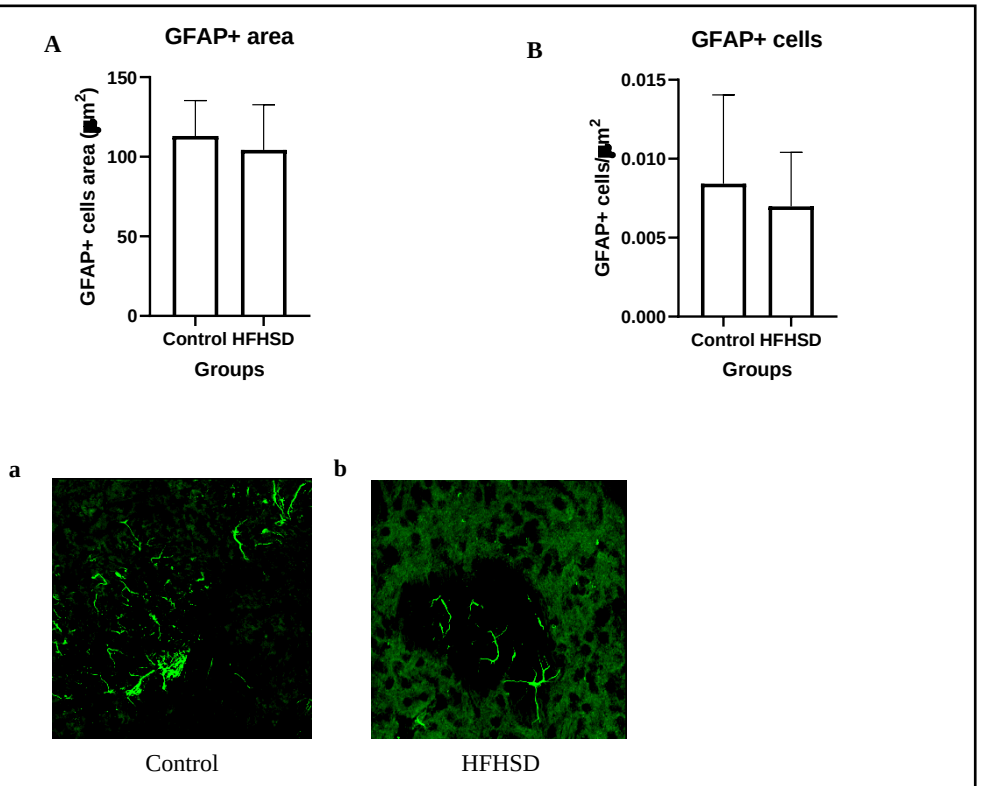

Figure 14: Immunofluorescence images of Control (a) and HFHSD (b) animal showing astrocytes expressing GFAP (Green). Area occupied by cells (A) and number of cells per square micrometre of area (B) within striatum of mice. n=4 animals per group.

Discussion

In this study, mice were given a diet rich in fat and a high-fat diet combined with high-sugar drink. We investigated synaptic degeneration, astrogliosis and microgliosis in the striatum. Furthermore, we also investigated any possible recovery upon diet reversal to low fat consumption.

The most significant observations were that levels of pre-synaptic proteins such as SNAP25 and Syntaxin-1 changed with HFD. SNAP25 levels decreased significantly for HFD60 and recovered for HFD-Rev whereas Syntaxin1 levels decreased for HFD60 but no recovery was observed upon diet reversal from high fat to control diet in HFD-Rev. Synaptophysin levels decreased significantly only for HFD45 but not HFD60. Altogether, these results indicate synaptic damage in the striatum of mice fed a fat-rich diet, in line with previous studies on the hippocampus (Lizarbe, Soares et al. 2018).

Synaptic proteins play a major role in maintaining synaptic plasticity which is essential for cognitive functions and most of these proteins are either directly involved in synaptic transmission and regulate synaptic function or serve as structural scaffolds (Rosenberg et al., 2014). SNAP25, an important SNARE protein helps in the fusion of neurotransmitter containing vesicles to membranes and regulates the plasticity of pre-synaptic neurotransmitter release (Gopaul et al., 2020). Under the influence of high-fat diet, the levels of SNAP25 gradually decreased with significant decrease in expression for 60% fat group followed by a recovery upon reversal of diet.

Syntaxin-1 levels also decreased in the 60% HFD group however no signs of recovery was observed upon diet reversal. Two isoforms of Syntaxin-1, namely syntaxin-1a (S1a) and syntaxin-1b (S1b), are present in neuronal membranes. S1b null mice show impaired Glutamatergic and GABAergic synaptic transmission, unlike S1a null mice which remain unaffected showing distinct functions of these isoforms in synaptic transmission in the CNS (Mishima et al., 2014). Recent studies conducted in syntaxin1b mutant models show an increased dopamine activity in ventral striatum due to reduced GABAergic transmission. The levels of GABA released in Ventral tegmental area reduced in S1b mutant model as investigated *in vivo* via micro dialysis studies enhanced dopaminergic activity in ventral striatum (Fujiwara et al., 2017). The decrease observed in syntaxin-1 levels in this study could point in a similar direction wherein DA circulation disruptions could be observed leading to impaired rewarding action of striatum. Syntaxin-1 expression level disruptions have also been reported in several neuropsychological disorders and S1b mutant models have shown anxiety-like behaviour and supressed BDNF levels which are all characteristic tendencies of Schizophrenia (Kofuji et al., 2014).

Post-synaptic proteins like Gephyrin and PSD95 showed a decrease in their expression levels for Reverse group. These proteins are essential for maintaining stability of synapses and anchor glutamate receptors.

Vesicular glutamate transporters (VGLUT 1-3) are selectively distributed on synaptic vesicles of excitatory glutamatergic terminals. VGLUT1 mediated alterations of such synapses in cortex and hippocampus showed abnormal behaviour in line with psychiatric and cognitive disorders (Tordera et al., 2007). Since striatum receives glutamatergic inputs from cortex, VGLUT1 and VGLUT2 levels were investigated. Neurons in the sub-thalamic nucleus of basal ganglia express VGLUT2 in majority and reduction of VGLUT2 mRNA within the region causes decreased post-synaptic activity and hyperlocomotion. This study conducted in mice showed decrease in preference for palatable food, altered behaviour along with impaired dopaminergic and peptidergic activity in striatum leading to opposing modifications in dorsal and ventral striatum due to a state of dysphoria (Schweizer et al., 2016). In this study, the levels of VGLUTs 1 and 2

show no significant changes which indicates absence of control over food intake and no visible alterations in synapses.

The Dopaminergic system of the mesolimbic circuit is known to be involved in reward-based behaviour. It is actively involved in food seeking and intake, addiction and mood disorders. Long term consumption of high calorie food impairs the dopaminergic system by reducing its sensitivity to insulin promoting diet induced obesity, decrease in levels of dopamine D_2 receptor signalling which is associated to weak impulse control increasing risk of addiction-like traits (Adams et al., 2015; Kleinridders et al., 2019). High-fat diets also impair Akt signalling and dopamine homeostasis in the striatum by altering Dopamine transporter cell surface expression (Speed et al., 2011). DAT1 levels showed tendency to decrease in both HFD (60%) and HFHSD. It was previously reported that 6 weeks of HFD feeding lead to a decrease in reuptake rates of dopamine in ventral striatum due to reduction in an immature isoform of DAT1 protein levels without any alterations in gene expression (Cone et al., 2013). This implies that dopamine reuptake from synapses and DAT-1 reduction levels are not due to decrease production of the protein itself but due to impaired trafficking or altered maturation owing to the impact of diet.

Another protein actively involved in the dopamine system is Tyrosine hydroxylase (TH) which is a rate-limiting enzyme in dopamine biosynthesis converting tyrosine to L-DOPA, precursor molecule for dopamine synthesis. TH is essential in regulating dopamine levels which in turn regulates movement and other locomotor functions. Reduction in dopamine signalling leads to increase in acetylcholine release from cholinergic neurons in striatum leading to movement disorders which are characteristic features of PD and Huntington's disease. TH levels along with TH-expressing neurons were reduced in the striatum and substantia nigra under the effect of HFD leading to defects in motor functions in open-field and vertical grid tests (Jang et al., 2017; Fiory et al., 2019). TH levels tended to increase in the striatum of mice fed the HFD for 6 months, while it tended to be reduced in mice fed the HFHSD for 1 month. A larger sample size would be necessary to confirm these results, but it suggests that either the presence of sugar in the diet, or the timing of diet exposure result in a biphasic TH modulation.

The striata from mice under HFHSD were stained for astrocyte and microglia markers GFAP and Iba1, respectively, in order to investigate the occurrence of gliosis. Studies conducted on rodent hippocampus and hypothalamus show an increase amount of GFAP+ astrocytes and Iba1 expressing microglia under the influence of a high-fat diet (Calvo-Ochoa et al., 2014; Baufeld et al., 2016). Even a short-term high fat, high fructose diet induced changes in microglia morphology and increased the number of reactive astrocytes in hippocampus of rats (Calvo-Ochoa et al., 2014). High fat feeding for 8 weeks showed significant increase in microglia and astrocytes in mice hypothalamus. Similar gliosis along with

microglia dystrophy was observed in hypothalamus of individuals with BMI>30 with most severe signs of dystrophy including shortened processes, enlarged cell body in obese individuals (Baufeld et al., 2016). Striatal GFAP and Iba1 cells significantly increased for Nrf2 deficient mice under the influence of Methamphetamine-induced toxicity. Nrf2 transcription factor regulates the redox potential in neurotoxin-induced oxidative stress. Under oxidative stress, dopaminergic neurons of nigrostriatal pathway undergo damage that ultimately affects motor functions (Granado et al., 2011). In our study, Iba1-positive microglia cells and their area tended to increase under HFHSD diet in relation to control mice. Although no significant changes were observed, the morphology of cells under diet showed an increase in number of bushy cells (short processes, enlarged cell body) and hyper-ramified cells (small cell body with more branching). The number and size of GFAP+ cells remained unchanged, which indicates the absence of astrogliosis. From the results above, it can be concluded that obesogenic diets are likely to cause microgliosis and neuroinflammation in specific regions of the brain but not in the striatum.

Conclusion

It was observed that synaptic proteins SNAP25, Synaptophysin and Syntaxin-1 decreased under HFD showing negative to some level of recovery upon reversal of diet. These proteins help in neurotransmitter release and play a role in reward modulation and control of motor activity. Despite any significant observations obtained from mice under obesogenic diet, a similar trend was followed with reduced DAT1 levels, slight increase in microglia occupied area and number of cells with altered morphology suggesting a shift from healthy to damaged striatal environment.
Further studies need to be conducted in HFHSD mice with a larger sample size along with a reverse group to investigate recovery of protein levels upon reversal of diet. The number of DAT1 and TH expressing neurons in striatum could be measured using immunohistochemistry, which is likely a better measurement of neurodegeneration than the amount of proteins. Levels of dopamine and distribution of dopamine receptors could also be analysed to investigate effective dopaminergic activity in striatum.

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