ISSN: 2714-4674 (Online)
ISSN: 2714-4666 (Print)

Annals of Clinical and Experimental Medicine

(ACEMedicine)

This Journal Is A Publication of
ASSOCIATION OF SPECIALIST MEDICAL DOCTORS IN ACADEMICS SOKOTO STATE CHAPTER

Volume 2, No. 1, January - June 2021

In this issue

www.acemedicine.asmeda.org

Effect of High-fat Diet on Neuron Density, Number and Nucleus Volume of Ventromedial Hypothalamus Nucleus: A Stereological Approach

Ahmed Muhammed Rabiu[1,2], Lawal Ahmed Abdulmumuni[3], Ali Ishaq Shugaba[4], Samuel Odu Odeh[5] and Nyengaard Jens Randel[1]
[1]Core Centre for Molecular Morphology, Section for Stereology and Microscopy, Department of Clinical Medicine, Aarhus University, Denmark.
[2]Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, Federal University of Lafia.
[3]Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, Federal University of Lafia.
[4]Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Jos.
[5]Human Physiology Department, Faculty of Basic Medical Sciences, College of Health Sciences, University of Jos.

Corresponding Author:
Ahmed Muhammed Rabiu
Core Centre for Molecular Morphology, Section for Stereology and Microscopy, Department of Clinical Medicine, Aarhus University, Denmark.
GSM: +234(0)80336650004
Email: rabiuam@yahoo.com

ACCESS TO THIS ARTICLE ONLINE

DOI: 10.47838/acem.26011977.127122021.asmeda.1.1

Website
https://www.acemedicine.asmeda.org

Abstract

Background: The hypothalamus is one of the best-studied and most important brain regions involved in the central control of feeding and energy expenditure from literature. The ventromedial hypothalamus (VMH) is designated as the principal satiety centre governing feeding behaviour, it is also considered to play a key role in regulating various behavioural and neuroendocrine functions. Obesity is now considered as a global epidemic by the World Health Organization. It has become more important because of the increased risk of mortality attributed to Corona Virus Disease 2019 (COVID-19) pandemic in persons with obesity. The study is aimed at analyzing the structural changes caused by high-fat diet in the VMH.

Materials and Methods: Twenty 4-week-old Wistar rats weighing between 37.8g to 69.3g were grouped into a control and a high-fat diet group. The control group were fed with standard rat chow and the experimental group with high-fat diet for 17 weeks. The animals were humanely euthanized (anaesthetized with Isofluorine in a glass chamber and euthanized with Natrium Pentobarbital (0.5 ml/kg body weight) via the intra-muscular (im) route) and the brain removed. The post-fixed brain tissue was sectioned with a microtome using systemic uniform random sampling (SURS) technique at a section thickness of 3µm and a section sampling fraction of 0.015. Sections were stained with toluidine blue. The neuron density, number and nucleus volume of VMH were estimated using unbiased stereological method.

Result and Conclusion: Long-term consumption of high-fat diet (HFD) insignificantly ($P>0.05$) decreased the numerical density of neurons and increased the volume of VMH in the hypothalamus. These findings may indicate that an increase in VMH volume and a decrease in neuron density and number maybe due to inflammation and gliosis caused by HFD as earlier reported.

Keywords: Ventromedial hypothalamus, Stereology, Systemic uniform random sampling, High-fat diet, Neuron number, Nucleus volume

Introduction

The brain is one of the most distinguishing features of the human species, and understanding it is one of the greatest challenges of twenty first century science. The Central nervous system (CNS) plays a key role in sensing and controlling the energy status of an organism (1), and the hypothalamus in particular has emerged as an integrating, superordinate master regulator of whole-body energy homeostasis. The hypothalamus is one of the best-studied and most important brain regions involved in the central control of feeding and energy expenditure.

It has been demonstrated that the type of diet consumed by an individual affects the brain structure and function (2). High-fat diet (HFD) may change the volume and neuronal number or density in the hypothalamus, which is the centre of energy control (3). It has been established that a lesion of the ventromedial hypothalamus causes hyperphagia and obesity (4). Although the exact physiologic mechanisms by which obesity adversely affects the brain are poorly understood, both experimental and human studies have revealed that brain function is sensitive to inflammatory pathways and mediators (5). Obesity has reached epidemic proportions across the developed and the underdeveloped world (6). Obesity is diagnosed when normalized for height, or body mass index (BMI) exceeds a defined threshold. People are said to be obese if their BMI exceeds 30kg/m^2. Above these BMIs (> 45kg/m^2, morbid obesity), more or less, the health risks of an increased weight become increasingly evident (7). Individuals with obesity (BMI > 40kg/m² or higher) have a higher risk of developing severe complication of COVID-19. (8), it is pro-inflammatory, and induces oxidant stress to adversely affect cardiovascular function (8).

The ventromedial nucleus of the hypothalamus (VMH) (fig., 1.0) is considered to play a key role in regulating various behavioural and neuroendocrine functions. For example, its role in feeding, aggressiveness (9), sexual behaviour (10) and gonadotropin release regulation (11, 12). The ventromedial nucleus of the hypothalamus (VMH) is designated as the principal satiety centre governing feeding behaviour (13). Established pathways involving orexigenic neuropeptide Y (NPY) and agouti-related polypeptide (AgRP) (NPY/ AgRP); and the anorexigenic pro-opiomelanocortin (POMC) and cocaine and amphetamine-related transcript (CART) (POMC/ CART) neurons project from the arcuate nucleus (AR) to other important hypothalamic nuclei, including the paraventricular (PVN), dorsomedial (DMN), VMH and Lateral Hypothalamus (LH) nuclei. In addition, there are many projections to and from the brainstem, cortical areas and reward pathways, which modulate food intake. Neuronal pathways between these nuclei are organized into a complex network in which orexigenic and anorexigenic circuits influence food intake and energy expenditure (14, 15).

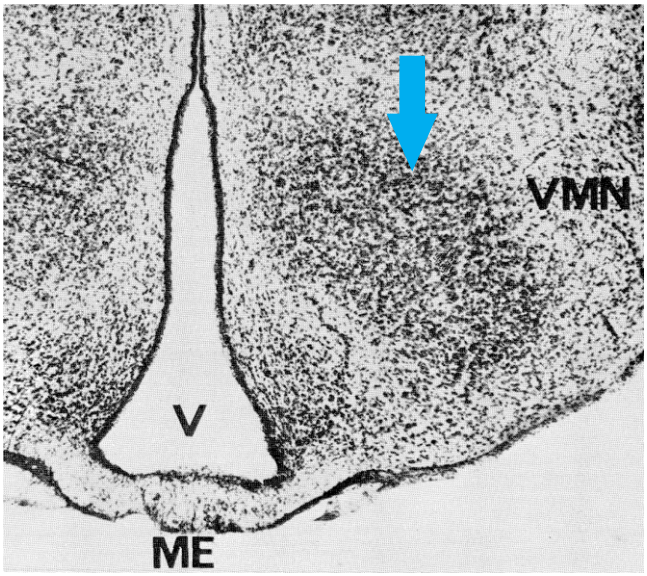

Figure 1: The Ventromedial Hypothalamus: vertical blue arrow showed a dense dark area (11)

As the hypothalamic nuclei are involved in the regulation of food intake, appetite control and energy balance, HFD may affect some or all of these nuclei, the study evaluated whether a long-term consumption of high-fat diet would change the numerical density and neuron number, as well as the volume of the ventromedial hypothalamus.

Materials and methods

Twenty Wistar rats of varying weight (37.8g – 69.3g) were obtained from the Laboratory Animal House of University of Jos at 4 weeks of age. Rats were housed in groups of five to acclimatize for two weeks prior to the study under 12-hour light/dark cycle, and were fed food and water *ad libitum*. All procedures in this study were conducted based on the guidelines of the Ethical Committee of Our Lady of Apostles Hospital (21052019), Jos, Plateau State.

The animals were humanely euthanized (they were anaesthetized with Isoflurane in a glass chamber and euthanized with Natrium Pentobarbital (0.5 ml/kg body weight) via the intra-muscular route). The brains of the rats were carefully removed from the skull using careful dissection. The post-fixed brain tissue was sectioned with a microtome (Microm, Model: RM2255, Leica Biosystems, Nussloch GmbH, Heidelberger Strasse 17 – 19, D-69226 Nussloch Germany) using systemic uniform random sampling (SURS) technique at a section thickness of 3µm and a section sampling fraction of 0.015. Sections were stained with toluidine blue. The neuron density, neuron number and nucleus volume of Ventromedial Hypothalamus were estimated using unbiased stereological method.

Experimental design

The two groups of rats (control and experimental groups, n = 10 per group) were provided with standard rat chow (control/normal diet; ND); MF nutrition plus (LF080119RF) and High-fat diet (Experimental diet, (HFD). The MF nutrition plus (HF080119RF) diet was obtained from Grand Cereal Jos, Plateau State, Nigeria) with water *ad libitum* for a period of 17 weeks to induce obesity (16).

Table 1: Constituent of the high fat diet

Constituent	g/Kg of diet
Casein	164
Corn starch	303.1
Dextrose	115
Sucrose	89.9
Butter oil	190
Cellulose	58.6
Soyabean oil	10
Mineral mix	41
Vitamin mix	11.7
L-cysteine	2.1
Choline bitartate	2.9

(Gaur *et al.,* 2014)

Histological and stereological studies

Brain tissue: Dissection and removal

At the end of the experiment (17 weeks), the rats were anaesthetized with Isoflurane in a glass chamber and euthanized with Natrium Pentobarbital (0.5 ml/kg body weight) via the intra-muscular route. The brains were carefully removed from the skull using careful dissection.

Histological studies

The brain tissue was fixed using Formaldehyde – 10% normal saline, 90ml distilled water (dH_2O), 0.9g NaCl for 24hrs. The tissue was then dehydrated with different concentrations of absolute alcohol; 50%, 70%, 80%, 90%, 95%, absolute I (100%), absolute II (100%)**.** It was then cleared (De-alcoholization) using xylene. Paraffin wax was then used to infiltrate the tissue to remove xylene, air and to fill micro holes (spaces) in the tissue. The tissue was finally Embedded or encased using paraffin wax and placed on plastic cassette. An embedding machine was used to pour a melted paraffin wax on the tissue that is in a rectangular steel mold, the plastic cassette was then laid on the mold and the paraffin wax was poured on it again and allowed to stand and solidify.

Sectioning: The brain tissue on the plastic cassette was mounted on a microtome holder and coronally sectioned using a Systemic Uniform Random Sampling (SURS) technique at a section thickness of 3µm, a section sampling fraction of 3/200 = 0.015 was used. The block tissue was intermittently place on a cold plate at -20^{0}c when it gets warm before cutting again, so as to a get a thin section (3µm). Cut sections were picked using a picker and placed into a warm bath (34^{0}c) guided by a paint brush. The section was strengthened out and picked using a glass slide (super frost – normal slide). It was then dried overnight in an oven at 36^{0}c.

Histochemistry Protocol

Toluidine blue was used as a nuclear staining agent after deparaffinization, dehydration and claring of the Formalin Fixated Paraffin Embedded (FFPE) tissue.

Stereological studies: The sections used for the estimation of the volume of the nucleus of interest (NOI - VMH) and the total number of neurons were selected at regular intervals of 200 µm, from the coronal sections containing the NOI, by applying a systemic random sampling procedure (17). The length of the hypothalamus from the beginning of the NOI was estimated to be 3200µm (18), the 15% shrinkage in length (19) due to the Formalin Fixated Paraffin Embedded (FFPE) treatment was also considered and a length of 2800µm was used. This length (2800µm) was divided by our cutting interval (200µm) that can cover the smallest NOI and a value of 16 series was arrived at. The first section was randomly sampled and the subsequent sections were systematically sampled – the 2nd and 3rd sections, which is equivalent to 9µm * 2 = 18µm (including the 1st section). Two consecutive/serial sections (dissection) were sampled and mounted on the same slide. One hundred and eighty-two (182) sections were then discarded before the next series sampling was commenced. However, the number of discarded sections was increased if the sections to be sampled were missed in the initial point of sampling. Care was taken to ensure that the sections were sampled consecutively within the same series. The section sampling fraction was 3/200 = 0.015. All the estimations were performed by using Visio pharm pathology software (VIS 2019.02, 2019.02.1.6005, visiopharm, DK-2970 Hoersholm, Agern Alle Denmark).

Light microscopy: A live modified research light microscope (Modified Research Light Microscope (Olympus DP70, Model BX 50F-3, Olympus Optical Co., LTD Japan) was used to view the sections on the slide at magnifications ranging from 10x to 60x, the microscope, connected to a camera (Olympus DP70 Digital microscope camera 12.5 megapixel, single chip color CCD, Japan) which transmits the microscopic image to a computer monitor, and an electronic microcator with digital readout (ND 281, Heidenhain, D-83301, 2006, Traunreut, Germany).

The volume was unbiasedly estimated by applying the principle of Cavalieri (20). In all selected sections, the profiles of the NOI - VMH were delineated at magnifications ranging from 10x to 20x across the sections. In each section, the cross-sectional area of the VMH was estimated by point counting with the use of a system of test points superimposed onto a computer monitor, in which the area per point (a/p) – equal to the area associated with one point in the grid, was calculated (a/p = *dx* x *dy* **/** magnification^2), the volume of the VMH was calculated from the total points that fell on it (recorded as sum of P = ∑P), and the distance between the systematically sampled sections (T) (21, 22). This value was calculated taking into account the section sampling fraction (ssf) and the section thickness (t).

V = T ***** (a/p) ***** ∑P, V (mm^3) = volume, T = t * 1/1/ssf, t = 3µm (0.003mm), ssf = 3/200 = 0.015, T = 200µm (0.2mm), a/p (µm^2) – was automatically generated by the software using the formula (a/p = *dx* x *dy* **/** magnification²).

The precision of the unbiased estimates of nucleus volume and neuron number was expressed by the coefficient of error (CE). The CE was calculated using the method by Gundersen *et al.,* 1999 (23). An average of 5 (±1) sections were counted per brain. A total approximate of 200 points was counted per nucleus analyzed. The mean CE of the estimates of the total volume and neuron number was 0.03 (3%) and 0.07 (7%) respectively. The Noise effect - the point counting variance, the Variance of ∑ area for Systemic Uniform Random Systemic (VAR_{SURS}) – a function of the number of sections and Coefficient of Variance is calculated using the formulas below:

Noise effect = 0.072 ***** (B/√A) ***** √(n∑P)

VAR_{SURS} (∑area) = $3(\sum(P_i * P_i) - \text{Noise}) - 4(\sum(P_i * P_{i+1})) + \sum(P_i * P_{i+2}) / 240$

Total variance of ∑P = Noise + VAR_{SURS} (∑area)

CE (∑P) = √(Total variance) **/** ∑P

Statistical analysis

Data obtained were expressed as Mean ± Standard error of mean (SEM). Independent student t test was used to analyze the data and to evaluate the significant difference, a P-value

< 0.05 was considered significant. All analyses were performed using IBM SPSS, version 26.0.

The precision of the individual estimates was evaluated as the coefficient of error (CE). The CE of the estimates of nucleus volume and neuron number were evaluated as a function of two independent factors: the noise effect and the variance due to sampling between systematically random sampled sections.

Results

Results for nucleus volume, neuron density and number in VMH: within the groups

Table 2.0 showed the coefficient of error (CE) to determine the precision for the calculations of the nucleus volume (0.03), the calculated CE is adequate for the sample slide used, Table 2.0 showed the coefficient of error (CE) to determine the precision for the calculations of the total neuron number (0.07), the calculated CE is adequate for the sample slide used.

Coefficient of error (CE) calculation: For precision determination.

Table 2: Estimation of the volume, V of Ventromedial hypothalamus (VMH) and the Coefficient of error, CE (∑P), of the estimate in an Individual[1]

Section	P_i	$P_i * P_i$	$P_i * P_{i+1}$	$P_i * P_{i+2}$
1	77	5184	7704	7632
2	114	11449	4708	4300
3	90	1936	748	0
4	30	289	697	0
5	0	1681	0	0
Sum (∑)	311	20539 (A	13857 (B	6791 (C)

a/p (mm^2) 0.002637
t (mm) 0.003
V (mm^3) = T * (a / p) * ∑P = 0.2 * 0.002637 * 311 = 0.164028
Noise effect = 0.164 * (BvA) * v(n∑P) = 0.164 * 8 * v(5*311)
= 0.164 * 8 * 39.4 = 51.74[a]
VAR_{SURS}(∑area) = 3(A – Noise) – 4B + C/240
= 3(27925 – 51.74) – 4 * 21738 + 10350/240 = 29.24[b]
Total variance of ∑P = Noise + VAR_{SURS}(∑area) = 51.74 + 29.24 = 80.98
CE (∑P) = √Total variance / ∑P = √80.98 / 311 = 0.03 (3%)

[1]Five sections were used in the analysis. Pi is the number of points counted on each of the 5 sections (i = 1 → 5). a/p is the area associated with each point.

[a]The variance of the point counting for each section was calculated according to Gundersen *et al.,* 1999 and the boundary/area ratio B/VA) was estimated to be 8 (17).

[b]The Systematic Uniform Random Sampling Variance (VAR_{SURS}) was calculated with the quadratic approximation formula, (23) taking into account the noise effect.

Table 3.0 shows the result from Ventromedial Hypothalamic Nucleus of nucleus volume, neuron density and neuron number within the groups for Wistar rats on normal and high-fat diet. The nucleus volume, neuron density and neuron number were not significantly different. The nucleus volume was marginally decreased and the neuron density and neuron number were decreased in the high-fat diet fed rats. The groups (ND, HFD) did not differ significantly, in Nucleus volume, t (11) = 0.50, P = 0.63, d = 0.03, 95% CI [- 0.05, 0.08], in Neuron density, t (11) = 0.53, P = 0.61, d = 83402.23, 95% CI [- 139435.83, 227698.31] and in Neuron number, t (7.2) = 1.65, P = 0.14, d = 9216.16, 95% CI [- 6448.00, 36890.30] (d = Standard Error Difference, CI = Confidence interval).

Table 3: Estimation of the Total number, Nv of Ventromedial hypothalamus (VMH) and the Coefficient of error, CE (∑P), of the estimate in an Individual[1]

Section	Q_i	$Q_i * Q_i$	$Q_i * Q_{i+1}$ –	$Q_i * Q_{i+2}$ –
1	85	7225	7565	3570
2	89	7921	3738	2403
3	42	1764	1134	0
4	27	729	0	0
5	0	0	0	0
Sum (∑)	243	17639 (A)	12437 (B)	5973 (C)

∑P 177
a/p = 7200/4 1800
t (mm) 0.003
V (mm^3) = T * (a / p) * ∑P = 200 * 1158.58 * 277
= 64185332 µm / 10^9 = 0.064185332 mm^3
Nv (mm^3) = ∑$\bar{Q}_i$ / t * (a/p) * ∑P = 243/3 * 1800 * 177 = 25806600
N = V * Nv = 0.064 * 25806600= 1651622.4
Noise effect = ∑$\bar{Q}_i$ = 243
Var(SURS, $\bar{Q}_i$) = 3(A – Noise) – 4B + C / 240
= 3(17639 – 243) – 4 * 12437 + 5973 / 240 = 35.05[a]
Total variance of ∑$\bar{Q}_i$ = Noise + VAR_{SURS}(∑area) = 243 + 35.05 = 278.05
CE (∑$\bar{Q}_i$) = √Total variance / ∑$\bar{Q}_i$ = √278.05 / 243 = 0.07 (7%)

[1]Five sections were used in the analysis. Qi is the number of neurons counted in the disector samples each of the 5 sections (i = 1 → 5).

[a]The Systematic Uniform Random Sampling Variance (VAR_{SURS}) was calculated with the quadratic approximation formula (23 and 21) taking into account the noise effect.

Table 4: Nucleus volume, Neuron density and Neuron number in VMH

Parameter	Diet	N	Mean ± [1]SD
Nucleus Volume (mm^3)	[3]ND	6	0.13 ± 0.04
	[4]HFD	7	0.12 ± 0.06
Neuron Density (x 10^5)	ND	6	2.96 ± 1.37
	HFD	7	2.52 ± 1.60
Neuron number (x 10^5)	ND	6	0.39 ± 0.20
	HFD	7	0.24 ± 0.10

Nucleus volume was not statistically significantly different, P = 0.63
Neuron density was not statistically significantly different, P = 0.61
Neuron number was not statistically significantly different, P = 0.14
[1]Stardard deviation

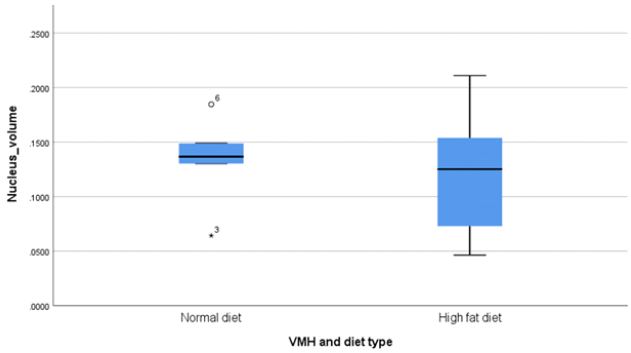

Figure 2: The Nucleus volume in Normal and High fat diet The graph showed an increase in the Nucleus volume with HFD

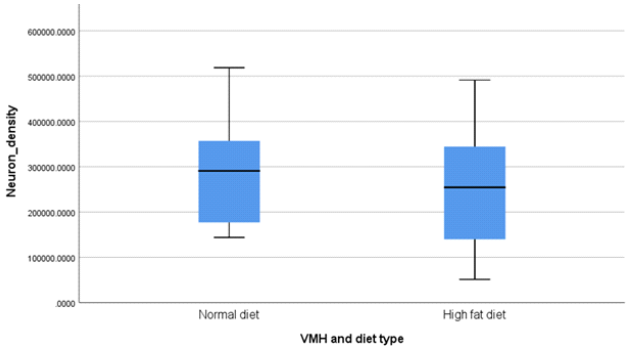

Figure 3: The Neuron density in Normal and High fat diet The graph showed a decrease in the Neuron density with HFD

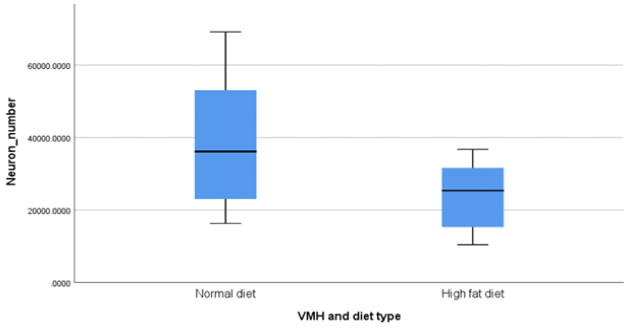

Figure 4: The Neuron number in Normal and High fat diet The graph showed a decrease in the Neuron number with HFD

Discussion

This is one of the first studies to our knowledge based on our literature search in Africa and among a few in the world evaluating the effects of high-fat diet on the nucleus volume, neuron density and neuron number of VMH. By using unbiased stereological technique, we showed that long-term feeding (17 weeks) of high-fat diet insignificantly increased the nucleus volume and decreased neuron density and number in VMH. A study by Reagear has reported that the volume of brain regions gives rather unspecific information about the function of that region, but structural changes may indicate a functional dysfunction of relevance taking place in that specific area (24). The increase in the volume of VMH in the hypothalamus in our study may be due to an increase in inflammation by high-fat diet, and an increase in the extracellular space as earlier reported (2, 3, 25). However further studies need to be carried out to ascertain this possibility. The reduction in neuron density is explainable by the fact that it is the number of neurons per unit volume and when the volume of the VMH in the hypothalamus is increased, neuron density decreases (since the number of neurons has not increased). The neuron number was also decreased following the high-fat diet feeding. However, these changes were noticed not to be of statistical significance when we analyzed the parameters within the nucleus of interest (VMH) using independent student t test. This increase in volume and decrease in neuronal density and neuron number following a high-fat diet may suggest an increased intercellular space by induction of inflammation or gliosis as has been earlier reported (2), neurogenesis in the hypothalamus (3) and increase apoptosis in the hypothalamus (25). Such changes can lead to a reduction in the activity of satiety center and energy expenditure signaling which can lead to an induction of obesity. The satiety centre contains neurons which carry receptors (leptin hormone receptors), a reduction in these neurons will indirectly decrease the availability of these receptors. These receptors are responsible for energy expenditure signalling and its impediment leads to obesity inducement.

Our calculated Coefficient of error (CE) to determine the precision of our estimates - nucleus volume, neuron number - was adequate (see table 1.0 & 2.0 respectively). The finding supports the idea that normal and high fat diet have an effect on neuron density and neuron number.

Conclusion

It can be concluded that long-term consumption of HFD decreases the numerical density of neurons and increases the volume of the VMH. These findings maybe as a result of inflammation and gliosis caused by HFD as earlier reported. Further investigation is however required.

Acknowledgment

We thank the Tertiary education Fund (TETFUND) for its financial support, Core Centre for Molecular Morphology, Section for Stereology and Microscopy, Department of Clinical Medicine, Aarhus University, Denmark for the use of its facility, Mr. Garba of the animal house unit University of Jos, Mr John Ben and Helene M. Andersen (Head of laboratory; Anatomy Department University of Jos and Core Centre for Molecular Morphology, Section for Stereology and Microscopy, Aarhus University, Denmark respectively).

Conflict of interest

There was no conflict of interest in the study.

References

1. Timper K., Bruning J.C. Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. Disease Models & Mechanisms 2017; 10: 679 – 689.
2. Pistell P.J, Morrison C.D, Gupta S, Knight A.G, Keller J.N, Ingram D.K, Bruce-Keller A.J. Cognitive impairment following high fat diet consumption is associated with brain inflammation. Journal of Neuroimmunology 2010; 219:25-32.
3. Namavar M.R., Raminfard S., Jahromi A.V, Azari H. Effects of high-fat diet on the numerical density and number of neuronal cells and the volume of the mouse hypothalamus: a stereological study. Anatomy and Cell Biology 2012; 45:178-184
4. Gaur A, Pal G. K, Ananthanarayanan P. H, Pal P. Role of Ventromedial hypothalamus in high fat diet induced obesity in male rats: association with lipid profile, thyroid profile and insulin resistance Annals of Neuro sciences 2014, 21: 104 - 107
5. Buettner R, Parhofer KG, Woenckhaus M, Wrede CE, Kunz- Schughart LA, Schölmerich J, Bollheimer LC. Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. J Mol Endocrinol 2006; 36:485-501.
6. World health organization (2020). Obesity and overweight. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (Last accessed 22/3/2020, 12:25pm)
7. Friedman J.M. A war on obesity, not the obese. *Science* 2003;299, 856 – 858
8. Centres for Disease Control and Prevention. People at higher risk for severe illness from COVID-19 2020; www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/people-at-higher-risk.html. (last accessed at 5:25am Apr., 06, 2020).
9. GBD 2015 Obesity Collaborators, Afshin A, Forouzanfar MH, et al. Health effects of overweight and obesity in 195 countries over 25 years. *New England Journal of Medicine*; 2017; 377, 13–27.
10. Hashikawa Y, Hashikawa K, Falkner A.L and Lin D. Ventromedial Hypo thalamus and the Generation of Aggression. Frontiers in Systems Neuroscience; 2017; 11:94 1 - 13.
11. Matsumoto A., Arai Y. Sex Difference in Volume of the Ventromedial Nucleus of the Hypothalamus in the Rat. *Endocrinology* Japon. 1983; 30 (3): 277-280
12. Christensen, L. W. and R. A. Gorski. Independent masculinization of neuroendocrine systems by intracerebral implants of testosterone or estradiol in the neonatal female rat. Brain Res. 1978; 146, 325-340.
13. Gaur A., Pal G.K., Ananthanarayanan P.H., Pal P. Role of Ventromedial hypothalamus in high fat diet induced obesity in male rats: association with lipid profile, thyroid profile and insulin resistance Annals of Neurosciences 2014; 21: 3104 – 107
14. Wang C, Godar RJ, Billington CJ, Kotz CM. Chronic administration of brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus reverses obesity induced by high-fat diet. Am J Physiol Regul Integr Comp Physiol 2010; 298: R1320-32.
15. Unger TJ. Examination of deficits in energy balance and affective behavior following central or hypothalamic depletion of brain-derived neurotrophic factor. Boston: Sackler School of Graduate Biomedical Sciences (Tufts University); 2007. p.194.
16. Mazor R, Friedmann-Morvinski D, Alsaigh T, Kleifeld O, Kistler E.B, Rousso-Noori L, Huang C, Li J.B, Verma I.M, Schmid-Schönbein G.W. Cleavage of the leptin receptor by matrix metalloproteinase–2 promotes leptin resistance and obesity in mice. *Science Translation Medicine* 2018; 10: 1-11.
17. Gundersen H.J.G., Jensen E.B. The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* 1987; 147, 229–263.
18. Paxinos & Watson. The rat brain in stereotaxic coordinates, 5th edition, *Elsevier*, Oxford UK 2004.
19. Dorph-Petersen K.A, Nyengaard J.R, Gundersen H.J.G. Tissue shrinkage and unbiased stereological estimation of particle number and size. *Journal Microscopy* 2001; 204: 232-246.
20. Cavalieri, B. (1635). Geometria Indivisibilibus Continuorum. Nova Quadam Ratione Promota. Bononiæ: Typis Clementis Feronij. Reprinted as Geometria degli Indivisibili. Unione Tipografico-Editrice Torinese, Torino, Italy, 1966.
21. Gundersen H.J.G., Bagger P., Bendtsen T. F., Evans S. M., Korbo L., Marcussen N., Moller A., Nielsen K., Nyengaard J. R., Pakkenberg B., Sorensen F. B., Vesterby A., West M. J. The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. Acta Pathologica, Microbiologica, et Immunologica Scandinavica APMIS 1988; 96: 857-881.
22. Madeira M.D., Sousa N., Santer R.M, Paula-Barbosa M.M, Gundersen H.J.G. Age and sex do not affect the volume, cell numbers, or cell size of the suprachiasmatic nucleus of the rat: An unbiased stereological study. *Journal of Comparative Neurology* 1995; *361:* 585–601.
23. Gundersen H.J.G., Jensen E.B.V., Kieu K., Nielsen J. The efficiency of systematic sampling in stereology—reconsidered. *Journal of Microscopy* 1999; 193(3): 199–211.
24. Reagear L. Increasing loss of brain tissue with increasing dementia: a stereological study of post-mortem brains from elderly females. *European Journal of Neurology* 2000; 7: 47–54
25. Moraes J.C, Coope A, Morari J, Cintra D.E, Roman E.A, Pauli J.R, Romanatto T, Carvalheira J.B, Oliveira A.L, Saad M.J, Velloso L.A. High-fat diet induces apoptosis of hypothalamic neurons. *PLoS One* 2009; 4: e5045.