Diet/photoperiod mediated changes in cerebellar clock genes causes locomotor shifts and imperative changes in BDNF-TrkB pathway

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Abstract

Neuropsychological studies report anxiety and depression like symptoms in patients suffering from lifestyle disorder but its impact on locomotor function lacks clarity. Our study investigates locomotor deficits resulting due to perturbations in cerebellum of high fat diet (HFD), chronodisruption (CD) or a combination (HCD) model of lifestyle disorder. Significant downregulation in levels of cerebellar clock genes (Bmal-1, Clock, Per 1 and Per 2) and Bdnf-TrkB pathway genes (Bdnf, TrkB and Syn1 levels and Erk1/2) were recorded. Further, locomotor deficits were observed in all the three experimental groups as evidenced by actimeter test, pole test and wire hanging test. Nuclear pyknosis of Purkinje cells, their derangement and inflammation were the hallmark of cerebellar tissue of all the three experimental groups. Taken together, this study generates important links between cerebellar clock oscillations, locomotor function and Bdnf-TrkB signaling.

Keywords
Locomotor deficits, Chronodisruption, Lifestyle Disorder, Neurobehavior, Bdnf-Trkb pathway, Neurodegeneration

1. Introduction

Frequent shifts in sleep-wake cycle coupled with shifts in timings of dietary intake have been implicated as one of the key causes for insulin resistance that culminates in lifestyle disorder\textsuperscript{1–3}. Increasing body of evidence implies to strong correlation between lifestyle disorder and mental health in populations with a history of consumption of high fat diet\textsuperscript{4–6}. Saturated fatty acids (SFAs) are instrumental in microglial activation and cytokine production (IL1B, IL-6, TNF-\(\alpha\)) that results in persistent low-grade inflammation, a characteristic feature of lifestyle disorder\textsuperscript{3,7}. Prolonged chronic inflammation is known to compromise functions by altering physiological processes wherein; a disrupted biological clock has also been identified as a key contributor\textsuperscript{8}. This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4747243
The master clock regulator resides in the Suprachiasmatic Nucleus (SCN) of mammalian hypothalamus and, several autonomous secondary clocks have also been reported from peripheral tissues and extrahypothalamic areas of the central nervous system\textsuperscript{9,10}. Internal clock of a cell is driven by an autoregulatory mechanism of molecular clock genes wherein, the core clock proteins BMAL1 and CLOCK heterodimerize to activate the downstream clock genes and other clock-controlled processes\textsuperscript{11–13}. In addition to that, the BMAL1-CLOCK heterodimer regulates the transcriptional-translation feedback loop by activation of Rev-erb-\(\alpha\) and Ror-\(\alpha\)\textsuperscript{13,14}. Therefore, Bmal1, Per1, Per2, Cry1, Rev-erb-\(\alpha\) and Ror-\(\alpha\) are not expressed constitutively but exhibit consistent circadian oscillations.

Dietary fat (>40\%) is known to cause pro-inflammatory changes in cerebral cortex (elevated TNF-\(\alpha\) and MCP-1 levels) and, triggers an early apoptosis in cerebellum of C56BL/6J mice\textsuperscript{15}. Such studies accredit overnutrition as a factor that affects cognitive and synaptic impairment\textsuperscript{16}. Likewise, an altered circadian cycle accounts for circadian desynchrony amounting to chronodisruption that is marked by alterations in molecular clock gene oscillations in key tissues such as the liver\textsuperscript{1}. Shift workers and trans-continental travelers are the prime group in this category likely to undergo photoperiod induced chronodisruption\textsuperscript{17,18}. Studies conducted on flight stewards had revealed depression and anxiety like behavior after frequent trans-continental travels\textsuperscript{19–21}.

Previous studies from our lab had reported diet/photoperiod induced altered biological rhythms of core clock genes in HepG2 cells and in liver \textsuperscript{1}. Likewise, neurobehavioral experiments on these models had provided compelling evidence on loss of cognition, depression and anxiety related behavioral perturbations \textsuperscript{4}. Available literature as well as finding from our lab establish diet as a factor responsible for neurobehavioral changes wherein; perturbation of Brain Derived Neurotrophic factor (BDNF)-Tyrosine Kinase (TrkB) pathway in various regions of the brain have been implicated as a causative factor. In this inventory, we hypothesize that diet/photoperiod induced altered status of clock genes in cerebellum of C57BL/6J mice causes locomotor deficits. Supportive evidence in form of altered expression of Clock genes, BDNF-TrkB pathway and histopathological changes are showcased in this study.

2. Materials & Methods

2.1 Analysis of Public Datasets

Public datasets consisting of High Fat diet fed cerebellum were retrieved from Sequence Read Archives (Gene Expression Omnibus: GSE62194; High Fat Diet: GSM1521816, GSM1521817, GSM1521818 and GSM1521819 and Control: GSM1521825, GSM1521826, GSM1521827 and GSM1521828), Reads from both the groups were compared using GEO2R and differentially expressed genes were identified. Analyzed data was downloaded with the adjusted \(p\)-value and log2FC. The differentially expressed genes were graphed using GraphPad Prism 9.0 heatmaps.
2.2 Experimental Animals

C57BL/6J male mice (40, 6-7 weeks old, 18-20 g) were purchased from ACTREC Mumbai and maintained as per CCSEA standard guidelines with laboratory chow and water ad libitum. Protocol was approved by the Institutional Animal Ethical Committee (IAEC) (No. MSU-Z/IAEC04/02-2020), and experiments were conducted in the CCSEA-approved animal house facility of the Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara (827/GO/Re/S/04/CCSEA). All the experiments were conducted as per ARRIVE guidelines. After 10 days of acclimatization to housing conditions, mice were segregated into 4 experimental groups (n=10/group) viz Group I (Control) mice fed on a laboratory chow diet; Group II (HFD) mice were fed on High Fat Diet (Supplementary Table 1); Group III (CD) Photoperiod Manipulation induced Chronodisruption (Supplementary Fig. 1); Group IV (HCD) mice were subjected to a combination of High Fat Diet and photoperiod Manipulation induced chronodisruption. Mice were subjected to the afore mentioned treatment regimes after 10 days of acclimatization. At the end of 17th week, mice were subjected to various neurobehavioral studies and euthanised at the end of 18th week (Supplementary Fig. 2).

2.3 Neuro-behavioral tests for locomotion

All tests were performed at diurnal ZT3 to ZT9 (i.e. 10-00 a.m. to 4-00 p.m.) timing. Briefly, the apparatus was cleaned with 70% ethyl alcohol and dried with a clean paper towel following each experiment or trial. During the observation periods, videos were recorded using a digital web camera (Logitech Webcam C270; 1280 x 720 pixels) connected to Dell Inspiron Laptop to ensure continuity of study. Video files were replayed and analysis was performed by neutral volunteers oblivious to experimental groups. Locomotor tests viz., Pole test, Beam walking test, Wire hanging test - for locomotory deficits, Light dark box test - for depression-like behavior were performed for control and experimental groups.

2.3.1 Locomotor counts

Rodent Actimeter (Model: ACT-01, Orchid’s Scientific, India) with clear square Plexiglas arena (50 cm × 50 cm) and equipped with 32- infrared sensors was used for the study. The infrared light beam interruption was measured (by automated counter) for 10 mins. Phenomenal clues/traits were cleaned (with 70% ethyl alcohol) between the test bouts.

2.3.2 Pole test (Locomotory deficits)

In the Pole test analysis mice were first placed (heads-up) on top of a wooden pole (50 cm long and 1 cm diameter.) A home cage was setup at the base of the pole. Mice tend to orient themselves downwards and descent the length of the pole back into their home cage. The time taken by the animal to orient downward ( t-turn) and total time to descent (t-total) were measured.
2.3.3. Wire suspension test (Locomotory deficits)

A metal wire (50cm long and 2mm thick) was tightly suspended 40 cm above the base of a frame. Mice were allowed to hang on (by their fore paws) to the wire and the latency to fall time was recorded with 180 sec losing the cut off time.

2.3.4. Beam walking test (Locomotory deficits)

In this test, the beam was divided into wider (12 mm) and smaller (8 mm) segments (1 m each). The tabletop was fastened to the beam (50 cm above) wherein; a black goal box was positioned at the narrow end of the beam. The count of time taken to reach to finishing line and the count of foot slips were video graphed for offline analysis. Home cage bedding was placed in the dark interior of the goal box to motivate the mice to enter the same.

2.4 Histopathological Study

At the end of the experiment, fasted overnight mice were euthanized (euthasol-150mg/kg, pentobarbital sodium-390 mg/ml and phenytoin sodium-50mg/kg) I.P. Whole brown was dissected out washed with 0.9% Phosphate buffered Saline, divided into three parts and stored in RNA-later (for gene expression study), 4% paraformaldehyde (for histology studies) or at -80°C (for protein studies).

Samples of cerebellum (n = 3/group) were dehydrated and embedded in paraffin wax blocks, and serial sections of 5 μm were cut using a microtome. Sections were stained with hematoxylin and eosin (H&E) or Cresyl Violet to observe gross histopathological changes. The same were photographed on a Nikon eclipse Ti2-E (Tokyo, Japan) microscope.

2.5 q-PCR Analysis

Total RNA content from cells and tissue were isolated using TRIzol reagent, and cDNA was synthesized using a iScript cDNA Synthesis kit (Bio-Rad, CA, USA). mRNA levels of candidate genes were quantified with qPCR (QuantStudio-5 real time PCR, Life Technologies, Carlsbad, CA, USA) using a SYBR Select Master Mix. The data were normalized with the internal control (18S or GAPDH) and analyzed using the 2−ΔΔCT method. A reaction tube w/o a template was used as a negative control, and all the samples were run with n = 3 technical replicates. Primer details are listed in (Supplementary Tables 2).

2.6 Immunoblot Analysis

Cells and tissue were homogenized in ice-cold lysis buffer with 1X protease inhibitory cocktail (Sigma Aldrich, USA). Total protein content was quantified using Bradford reagent (Bio-Rad, USA). Equal amounts of protein from each sample were loaded on 10% gel for SDS-PAGE. Protein was further transferred to PVDF membrane (Bio-Rad, USA) and blocked with 5% skimmed milk (HiMedia) following overnight incubation in primary antibody (BMAL1-1,
1:1000; BDNF, 1:1000; Clock, 1:1000; TrkB, 1:1000; SYN1, 1:1200; ERK1/2, 1:1500) prepared in 3% BSA. Secondary antibody (anti-rabbit, 1:1000; anti-mouse, 1:5000) treatment was done for 1 h, and blots were developed using ECL reagent. The membrane was stripped using stripping buffer and was re-probed with β-actin (1:1000).

3. Results

3.1 Diet/Photoperiod induced perturbation disrupts the cerebellar clock gene expression.

In order to gain insights into the effects of HFD and Bmal1-/-on cerebellar transcriptome, we looked at publicly available datasets on Gene Expression Omnibus and Sequence Read Archives (SRA). We had re-analysed the raw data using GEO2R & Galaxy RNA Sequencing pipeline. Bmal1, Clock and BDNF were found to be differentially expressed in cerebellum of HFD fed mice but, Per1, Per2 and Rev-erb-α did not show any significant change (Fig. 1A). In order to strengthen our claim, we had checked the perturbation in cerebellar clock in our experimental groups viz. HFD, CD and HCD. Transcripts of Bmal-1 (**** p< 0.0001) (Fig. 1B), Clock (* p< 0.05) (Fig. 1C), Per1(*** p< 0.0002) (Fig. 1D) and Per2 (** p< 0.001) (Fig. 1E) were significantly downregulated in all the treated groups as compared to the control group. These results suggest that, both high fat diet as well as photoperiodic changes are able to upset the cerebellar molecular clock circuitry.
Figure 1: Diet/Photoperiod manipulation induced chronodisruption in cerebellum of C57BL/6J mice. Results of differentially expressed genes in high fat diet fed cerebellum of publicly available datasets (A). The mRNA levels of cerebellar clock genes viz. Bmal1 (B), Clock (C) Per1 (D) Per2 (E), Cry1 (F), Cry2 (G), Rev-erb (H) and Drp (I). Data represented as Mean ± SEM *p<0.05, **p<0.01, ***p<0.001, ****p< 0.0001 vs Control.

3.2 Diet/Photoperiod induced perturbations account for locomotor shifts.

Before termination of the protocol, possible deficits in locomotor or motor coordination were assessed by animal actimeter test (Fig. 2A), pole test (Fig. 2B), wire hanging test (Fig. 2C) and beam walking test (Fig. 2D). Actimeter test showed significant downregulation in locomotor counts in CD group (* p<0.05) and a non-significant decrement in HFD and HCD groups. In pole test, the mice subjected to CD and HCD (* p<0.05) took significantly more time to descend down from the pole, but HFD (* p<0.05) and HCD (** p<0.01) mice took significantly more time to turn implying towards subtle changes in locomotor function. Observations of the wire hanging test were in conjunction with previous results wherein; HFD (* p<0.05) and HCD (** p<0.01) mice recorded significantly higher average fall counts compared to control. Beam walking test did not show any significant change with CD mice recording a non-significant
increment. It is interesting to note that HFD and HCD groups recorded similar results suggesting that the diet has a profound effect on cerebellar function that is more compared to experimentally induced chronodisruption.

**Figure 2:** Diet/Photoperiod induced perturbations accounts for locomotor shifts in C56BL/6J mice. Locomotor counts (A), Average time to descend (B), Average time to turn (B), Average fall counts (3 mins) (C), Time to cross beam (E). Data represented as Mean ± SEM *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs Control.

3.3 Microscopic evaluation of cerebellum shows pathophysiological changes due to Diet/Photoperiod induced perturbations.

Hematoxylin Eosin (H&E) (Fig. 3A) and Cresyl Violet (Nissl) staining (Fig. 3B) of paraffin embedded sections (n= 4to5) of cerebellum were subjected to microscopic observations. H&E staining showed visible gaps between molecular and granular layers possibly due to Purkinje cell shrinkage in HFD, CD and HCD groups. Further, distorted Purkinje cell (arrow) and gross pyknotic changes in granular or molecular layers were observed in all the three experimental groups. Inflammation and immune cell invasion (*) were also evident in these groups.
3.4 Alterations in cerebellar BDNF-TrkB pathway correlates with changes in locomotor behavior.

The mRNA (Fig. 4 A-F) and protein levels (Fig. 4 G-J) of the candidate genes of cerebellar Bdnf-TrkB pathway were studied to decipher the neuronal survival and plasticity. Briefly, the Bdnf, TrkB and Syn1 transcripts were significantly downregulated in all the three experimental groups whereas; Psd95 showed a non-significant decrement. The Nt-3 and Nt-4 transcripts were seen to be significantly downregulated in HFD and CD groups but a significant increment in HCD group (** p<0.001) was an offshoot result. Immunoblots of Bdnf and TrkB were significantly downregulated in CD and HCD groups but showed no change in HFD group. Syn1 was significantly downregulated in all the treatment groups. Erk1/2 is a downstream protein activated by Bdnf/TrkB signaling and the same was significantly upregulated in HFD (**** p<0.0001). Lowered levels of Erk1/2 in HCD (* p<0.05) and no change in CD group were contrary to the expected results. Involvement of Erk 1/2 in several apoptotic pathways can be a possible cause of the observed negative result in CD and HCD groups. 

Figure 3: Microscopic evaluation of cerebellum shows pathophysiological changes due to Diet/Photoperiod induced perturbations. Representative images of Haematoxylin & Eosin (A) and Cresyl Violet (B) stained sections observed at 40x magnification. H & E (A) stained representative images of Control (i), HFD (ii), CD (iii), HCD (iv). Cresyl Violet (B) stained representative images of Control (i), HFD (ii), CD (iii), HCD (iv).
Figure 4: Alterations in cerebellar BDNF-TrkB pathway correlates with changes in locomotor behavior. The mRNA levels of Bdnf-TrkB pathway genes Bdnf (A), TrkB (B), Syn1 (C) PSD 95 (D), Nt3 (E) and Nt4 (F). Their representative immunoblot Bdnf (G), TrkB (H), Syn1 (I) and Erk1/2 (J). Data represented as Mean ± SEM *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs Control.

4. Discussion

The circadian clock gene oscillations in mammalian brain are regulated by the Suprachiasmatic nucleus (SCN) neurons of hypothalamus wherein, the molecular clocks operate by autoregulatory loops. Amongst various cerebral clocks operating outside the master clock, the cerebellar region shows a distinct 6-hour delay as compared to SCN. The cerebellar cortex of mouse exhibits a robust rhythm as evidenced by the clock gene transcripts. This is despite no direct neural pathway between the SCN and the cerebellum. Rath et. al. (2012) had given a comprehensive account of 8 core clock genes in rat cerebellum viz. Per1, Per2, Per3, Cry1, Cry2, Rev-erb-α, Bmal1 and Clock. Seven of these genes were known to show periodic oscillations but clock was reported to have an arhythmic expression. This study had also
emphasized on the regulatory role of SCN vis-à-vis cerebellar rhythm\textsuperscript{26}. Since phase shifting effects of restricted feeding had revealed that cerebellar clock gene oscillator can be entrained to mealtime \textsuperscript{27}, in our study we had investigated the expression of core clock genes in cerebellum of High fat high fructose diet (HFD) fed C57BL/6J mice. Also, phase advance/phase delay photoperiodic shifts amounting to chronodisruption (CD) was studied as another experimental group. A combination of HFD+CD (HCD) was studied to emulate a real life scenario of lifestyle disorder. In first phase of the study, transcripts of core clock genes were assessed in the cerebellar tissue wherein; the Bmal1, Clock, Per1, Per2, (Fig. 1B-E) showed a significant decrement in both HFD and CD treated groups. A previous study from our lab had reported similar set of changes in hepatic clock genes and, the observations in cerebellar tissue are in agreement with these findings\textsuperscript{1}.

Any form of manipulation in the standard composition of dietary macronutrient (protein, fat or carbohydrate) in experimental rodents has been implicated to affect behavior and spontaneous activity\textsuperscript{28}. The decrement in locomotor activity and motor coordination in HFD mice observed in our study is in agreement with the published reports. Further, our observations also imply towards a possible aberrations in motor function. Likewise, the results obtained in CD group had a similar pattern as the one observed in HFD group. It is interesting to note that HCD group too, showed similar set of changes that can be attributed to diet and/or photoperiod induced locomotor deficit; a reflection of multi-factorial aetiology seen in metabolic disorder.

Obesity induced metabolic dysfunction is associated with neuronal death that culminates in impairment of cognitive function. These pejorative changes have also been implicated in elderly people. Growing body of evidence suggests that high fat diet can cause early aging associated neurodegenerative changes\textsuperscript{29}. Photoperiodic manipulations and circadian desynchrony have been broadly implicated to cause behavioral changes in humans and experimental models. But, effect of photoperiodic manipulation induced chronodisruption and its impact on locomotor function lacks clarity. In our study, histopathological implications on cerebellum of HFD, CD or HCD groups has shown varying degrees of qualitative changes in neuronal morphology in the cerebellar cortex. Munos-Castaneda et. al. 2018 had reported that motor coordination is impacted only after loss of Purkinje cells \textsuperscript{30}. Distorted shrunken electron-dense Purkinje cells along with vacuolation or shrinkage of neurons in the granular and molecular layer of cerebellar cortex are the hallmark that translates into locomotor deficits. The observed distorted Purkinje layer and vacuolation are in agreement with the published reports and in H x E and Creysl Violet stained cerebellar cross-sections of HFD, CD and HCD groups compliment the deficits recorded in locomotion and motor coordination.

Brain Derived Neurotrophic Factor (BDNF) has been reported by several research groups as a regulator of neuronal survival and morphology \textsuperscript{31}. BDNF is a growth factor released into circulation and works in conjunction with its receptor TrkB. BDNF is centrally involved in regulating long term synaptic potential and strength \textsuperscript{31–33}. Literature suggests that activation of BDNF attenuates symptoms of neurodegeneration and cognitive deficits. Dysregulation of BDNF-TrkB signaling is known to activate ERK1/2; a critical step in BDNF signalling cascade. Bomba et. al. (2018) has identified negative role of ERK1/2 due to its involvement in
several apoptotic pathways. Our findings showcase a significant decrease in BDNF, TrkB and Syn1 in cerebellar tissue of HFD, CD and HCD groups that provide evidence for locomotor deficits recorded in our study. Taken together, the loss of synaptic plasticity and shrunken Purkinje cells can be attributed to the changes in cerebellar Bdnf-Trkb pathway culminating in locomotor deficits. This inventory adds a new dimension to our understanding of diet/photoperiod induced changes in locomotor function and warrants detailed investigation to decipher aging/lifestyle disorder relates changes in cerebellar function.

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Abstract

Neuropsychological studies report anxiety and depression like symptoms in patients suffering from lifestyle disorder but its impact on locomotor function lacks clarity. Our study investigates locomotor deficits resulting due to perturbations in cerebellum of high fat diet (HFD), chronodisruption (CD) or a combination (HCD) model of lifestyle disorder. Significant downregulation in levels of cerebellar clock genes (Bmal-1, Clock, Per 1 and Per 2) and Bdnf-TrkB pathway genes (Bdnf, TrkB and Syn1 levels and Erk1/2) were recorded. Further, locomotor deficits were observed in all the three experimental groups as evidenced by actimeter test, pole test and wire hanging test. Nuclear pyknosis of Purkinje cells, their derangement and inflammation were the hallmark of cerebellar tissue of all the three experimental groups. Taken together, this study generates important links between cerebellar clock oscillations, locomotor function and Bdnf-TrkB signaling.

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