Synopsis of the thesis entitled

Circadian and epigenetic control of Nocturnin in Non-alcoholic steatohepatitis

Submitted to

The Maharaja Sayajirao University of Baroda
Vadodara-390002, Gujarat, INDIA

For the Degree of Doctorate of Philosophy

In

Zoology

Ph.D. Student

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Synopsis of PhD Thesis

September, 2023

Introduction:

Non-alcoholic Fatty Liver Disease (NAFLD) is being increasingly recognised as a common liver disorder which represents the hepatic manifestation of the metabolic syndrome, a variably defined aggregate of disorders related to obesity, insulin resistance, hypertension and hyperlipidaemia (Brunt et al, 2004). Epidemiological studies report the prevalence of NAFLD in 9% to 32% of the general Indian population (Duseja et al, 2010) with the global prevalence being 25% (Younossi et al, 2016), making it a major health concern. Non-alcoholic steatohepatitis (NASH) is the progressive form of NAFLD which carries the risk of progressive fibrosis, cirrhosis, and end-stage liver disease (Reid et al, 2001). NASH etiology can be characterised by excessive triglycerides (TGs) and free fatty acid (FFA) accumulation in the liver which subsequently leads to inflammation, apoptosis, and ballooning degeneration (Thounaojam et al., 2012).

One of the major factors contributing to the development of NAFLD and its subsequent progression to NASH is circadian disruption. Studies provide a strong link between circadian rhythm disruption and the onset of a variety of human diseases. Desynchrony of circadian rhythms and the external environment, such as shift work, chronic jet lag, intentional sleep restriction, deprivation and night eating can markedly contribute to increased morbidity. Shift workers for example, exhibit a higher prevalence of obesity and associated disorders, such as NAFLD (Johnston *et al*, 2014). A connection between the circadian rhythm and NAFLD has relatively recently been proposed (Gnocchi *et al*., 2015). All the mechanisms involved in NAFLD pathogenesis and its evolution to NASH are under regulation of the molecular clockwork. This includes endoplasmic reticulum (ER) stress, lipotoxicity, insulin resistance, mitochondrial dysfunction, oxidative stress, adipose tissue dysfunction, deranged control of innate immunity, and cytokine secretion as well as alterations in the gut–liver axis (Shetty *et al,* 2018).

The circadian clock regulates hepatic lipid metabolism via the circadian clock gene output. One of the recently discovered circadian clock output gene is *Nocturnin* (*Noct)* or *Ccrn4l.* Although Noct is not directly involved in regulating core clock gene expression, it does have functions in mediating its rhythmic output. Because many of the rate-limiting enzymes in metabolic reactions are under circadian control, gene expression must be tightly controlled and post-transcriptional mechanisms such as deadenylation play an important part in this process. Noct, as a circadian deadenylase, is poised to play an important role in post-transcriptional regulation of metabolic genes under circadian control.

In healthy mice, hepatic *Noct* expression is known to peak between ZT12-ZT18 which corresponds with lowered expression of hepatic lipid metabolism genes. Mice subjected to ad-libitum high fat diet (HFD) feeding for 3 weeks revealed an increased expression and amplitude of hepatic *Noct* mRNA with a peak at ZT12 as compared to the control; suggesting that chronic HFD consumption can alter *Noct* rhythmicity in liver (Stubblefield et al., 2018; Kulshrestha S. & Devkar, R., 2023). The *Noct* KO mice showed an increased expression of metabolic genes (*Acly, Hmgcs1, Pmvk, Mvd, Sqle, Dhcr7, Acaca, Fasn, Pnpla3, Fdps* and *Gpam)* at ZT18 as compared to WT mice thus underlining the importance of NOCT as a regulatory of hepatic metabolism (Stubblefield et al., 2018; Kulshrestha S. & Devkar, R., 2023). Also, these *Noct* KO mice fed with HFD did not record any significant body weight changes and respiratory exchange ratio like the WT mice that can be attributed to the observed upregulation of genes governing hepatic metabolism in absence of NOCT (Stubblefield et al., 2018; Kulshrestha S. & Devkar, R., 2023). These findings are suggestive of the significance of Noct in regulating hepatic lipid metabolism, yet its exact role in the pathophysiology of diseases like NAFLD and NASH.

Since *Noct* is robustly expressed in the liver and is involved in regulating the hepatic metabolism, its expression must be tightly regulated by several non-coding RNA molecules like micoRNAs (miRNAs). Among the numerous miRNAs which are involved in the maintenance of liver function, miR-122 is one of the liver-specific miRNAs regulating *Noct* expression (Kojima *et al*, 2010). miR-122 is a paradigm for the role of miRNAs in the liver (Girard et al, 2008). Functional studies on this miRNA showed that miR-122 is involved in cholesterol biosynthesis (Krützfeldt et al, 2005), fatty acid synthesis and oxidation (Esau et al, 2006). Despite the available evidence, it is still unknown whether *Noct* functions directly or indirectly in regulating the metabolic processes in the liver. Since, *Noct* is also a target of miR-122, which could be major contributing factor in the pathophysiology of NASH, it will be interesting to study the miR-122: Noct axis in the aforementioned pathology. This axis could further provide evidence on the mechanistic link between the circadian clock disruption and the onset and progression of metabolic anomalies like NASH.

Baseline studies from our lab have shown that interactions between an altered energy metabolism and disruptions in the circadian clock creates a downward spiral culminating in NASH wherein; the circadian oscillations of core clock genes were found to be altered and later corrected by exogenous melatonin administration (Joshi *et al*, 2021). Our lab has also reported elevated miR-122 expression in the circulation under conditions of NASH, which was in

alignment with various other studies. Further, our *in silico* docking studies have also confirmed *Nocturnin* as one of the predicted targets of miR-122, again corroborating with the available literature. *Noct,* along with being a clock output gene, also has important regulatory roles in lipid and cholesterol metabolism in the liver and alterations in its expression could have significant consequences on the pathophysiology of NASH, which has not been yet investigated in detail. It shall be remarkable to assess the merits of the miR-122: Noct axis in the liver under the conditions of the aforementioned pathology. Further, we hypothesize a possible role of Melatonin in altering the miR-122: Noct axis and their downstream genes involved in lipid metabolism and implicated in NASH. In order to envisage the systemic model of lifestyle disorder, evaluating the circadian and the epigenetic basis of Noct shall also provide crucial evidence for painting a holistic picture of role of *Nocturnin* and miR-122 in said lifestyle disorder.

Hypothesis

Alterations in biological clocks coupled with high calorie diet forms the basis of a majority of the lifestyle disorders including NASH. miRNAs have been recently shown to play a key role in modulating and controlling several cellular functions; amongst these, miR-122 is well-studied as a liver-specific miRNA. Circadian basis of NASH has been variedly reported by research groups including the results obtained from the previous studies conducted in our lab (Joshi *et al.,* 2021). It is hypothesized that Noct, especially under chronodisruptive conditions may play a putative role in NASH pathophysiology. Changes in miRNA and altered synergy of metabolic genes in circadian dysregulation are imperative for the said pathophysiological changes in NASH. Since, miRNA: gene networks are crucial but are minimally explored in NASH, we hypothesize a miRNA: Noct axis as a key pathway instrumental in the pathophysiology of NASH. Further, we hypothesize possible interactions between melatonin and Noct functioning in the pathophysiology of NASH.

Key Questions

1. In what way chronodisruption (CD) alter hepatic Noct expression?
2. How does an altered miRNA profile affect Noct expression in NASH?
3. What is the role of Noct in the pathophysiology of NASH in the liver?
4. Does melatonin have any possible effect on Noct expression in CD and/or HFHF induced NASH?

Objectives:

Objective 1. To evaluate circadian basis of Nocturnin in high fat high fructose (H) and/or photoperiodic manipulations induced chronodisruption (CD) in pathology of non-alcoholic steatohepatitis (NASH).

Study 1. In silico functional analysis of Nocturnin.
Study 2. *In vitro* model generation of NAFLD in HepG2 cells and studying Noct oscillations.
Study 3. *In vivo* model generation of H and/or CD induced NAFLD in C57BL6/J mice and assessing hepatic Noct oscillations.

Objective 2. To investigate the epigenetic regulation of Nocturnin in CD induced NASH.

Study 1. An *in silico* approach to investigate the role of an altered miRNA profile in regulating *Noct* expression in NASH and identifying key candidate miRNAs.
Study 2. Studies in HepG2 cells to validate miRNA: *Noct* association in NASH.
Study 3. Establishing association between the identified miRNA and *Noct* in H and/or CD induced NASH in *in vivo* animal model.

Objective 3. To investigate the possible melatonin vs Nocturnin synergy in H and/or CD induced NASH.

Study 1. Molecular docking studies of melatonin and NOCT.
Study 2. Assessing the possible role of melatonin in improving Noct oscillations in HepG2 cells.
Study 3. Assessing the possible role of melatonin in improving hepatic Noct oscillations in HFHF and/or CD induced NASH pathology.

Observations:

Objective 1. To evaluate circadian basis of *Nocturnin* in CD induced NASH pathology.

Study 1. Functional analyses of Nocturnin

Nocturnin is a recently discovered circadian clock output gene with a robust rhythmic expression in the liver. Despite the available literature, there is still an ambiguity about the role of Noct in the liver, especially under pathologies like NASH. We devised an *in silico*

methodology to understand the same. Gene Expression Omnibus (GEO) datasets of control and *Noct* KO A549 cells were retrieved from NCBI (GSE123477) and analysed in GEO2R. Out of 17,428 genes, 71 genes were found to be differentially expressed (DEGs) in *Noct* KO cells. These 71 genes were further analysed in Search Tool for the retrieval of Interacting Genes/Proteins (STRING) database and visualized in Cytoscape 3.9.1. Subsequently, we performed gene ontology enrichment analyses in Webgestalt wherein, lipid metabolism pathway genes were significantly upregulated in *Noct* KO cells, suggestive of the role of Noct in regulating lipid metabolism.

(A) (B) (C)

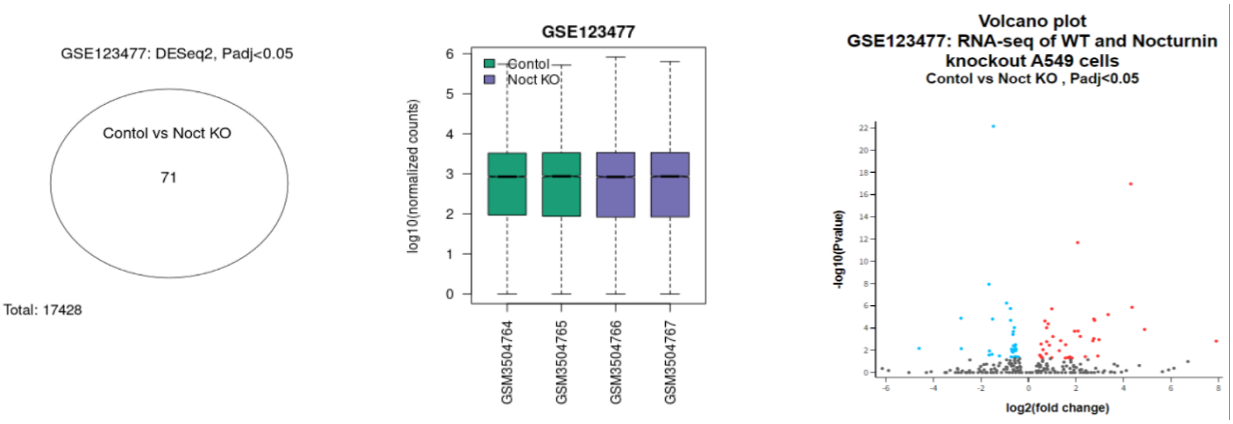


Figure 1. Screening of the DEGs: (A) Venn diagram of the differentially expressed genes in Control and Noct KO cells; (B) Box plot of the GSE123477 dataset and (C) Volcano plot of the GSE123477 dataset: blue dots indicate downregulated genes while the red dots indicate upregulated genes.

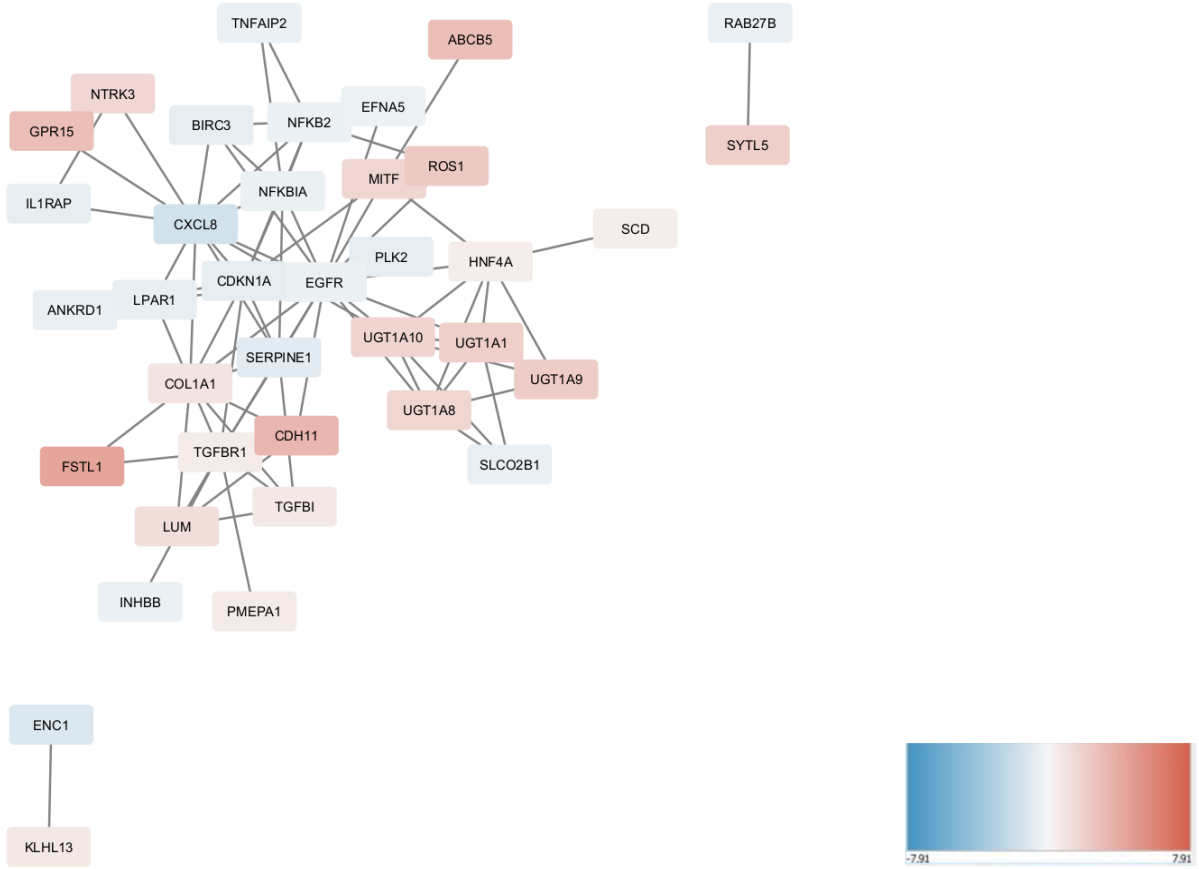


Figure 2. Protein-protein interaction network of the DEGs.

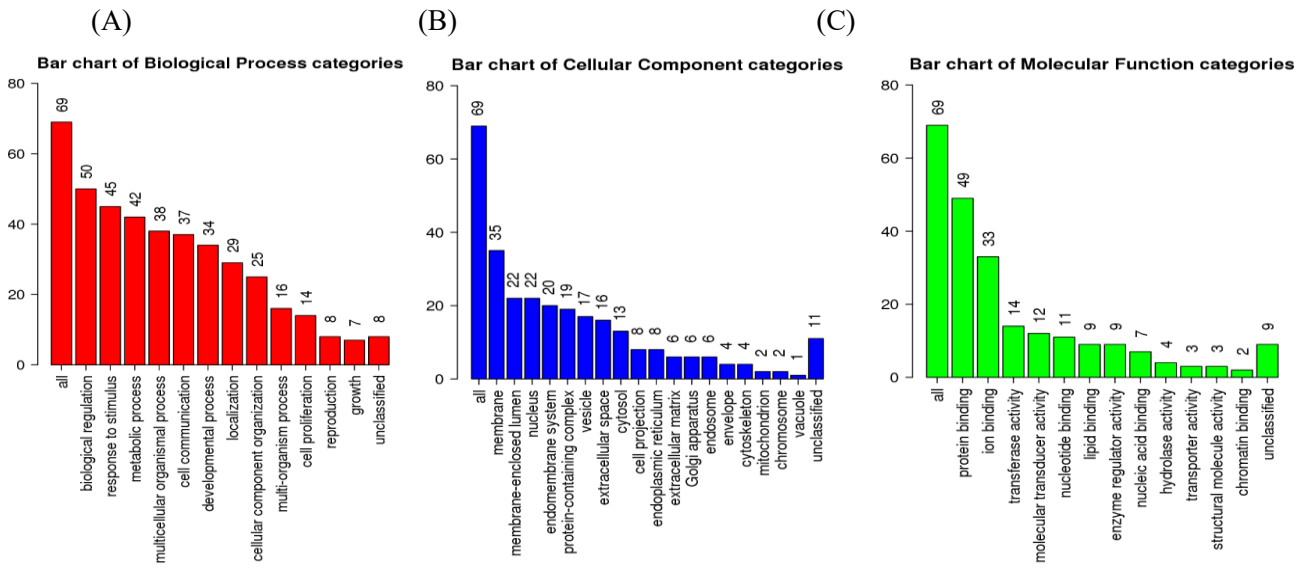


Figure 3. Enrichment of the DEGs in (A) Biological processes, (B) Cellular Component and (C) Molecular Function categories, considering adj p-value < 0.05.

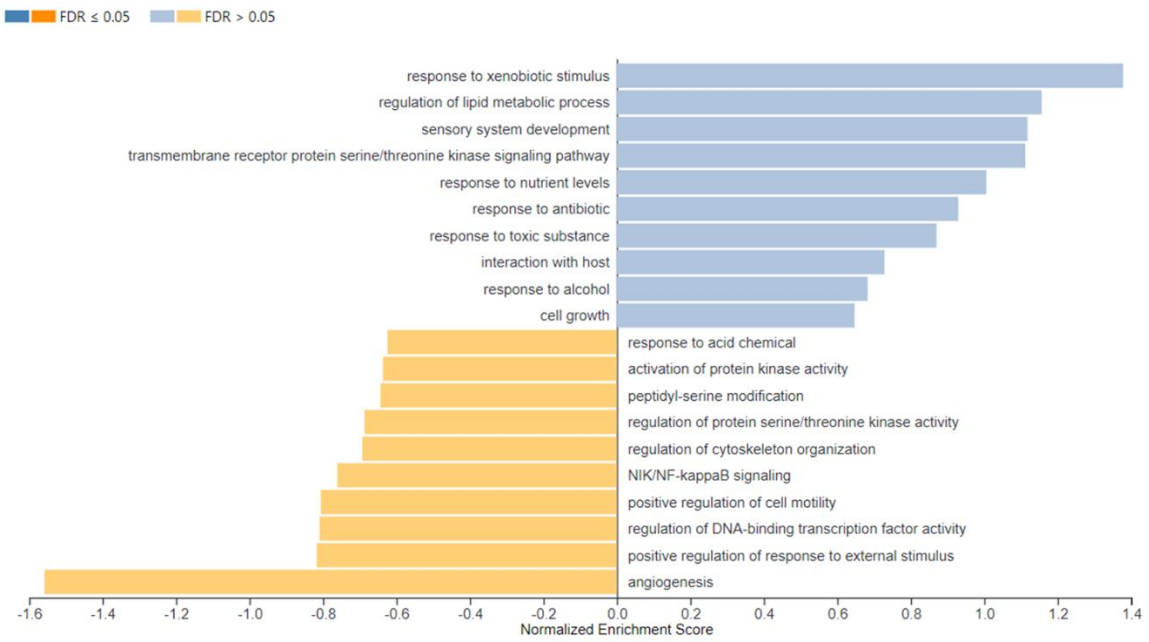
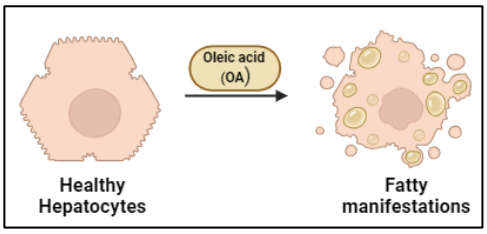


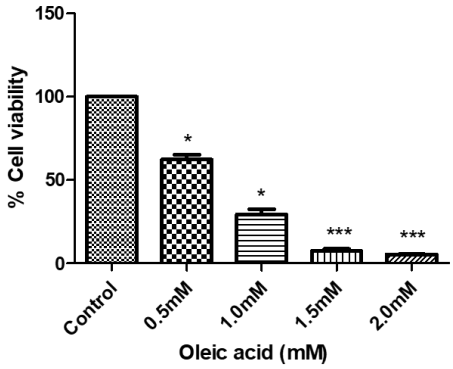
Figure 4. Summary of Enrichment analysis of the DEGs.

Study 2. *In vitro* model generation of NAFLD in HepG2 cells and assessing Noct oscillations.



Oleic acid (OA) was used to induce fatty manifestations in HepG2 cells and generate an *in vitro* model of NASH. Treatment with OA (0.5 -2 mM) caused a dose dependent decrement in percentage cell viability in MTT cytotoxicity assay. From the results of MTT assay, 0.5 mM dose of OA was selected for further experiments. The validation of this dose was further done by performing Oil Red O (ORO) staining wherein, 0.5 mM OA treatment for 24 hours caused a significant accumulation of lipids in the HepG2 cells.

(A)



(B)

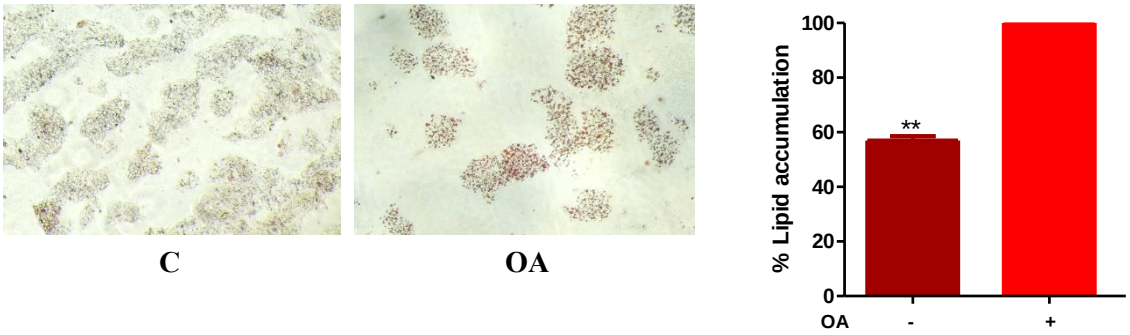


Figure 4. (A) Cytotoxicity (MTT) assay to standardize the dose of OA in HepG2 cells; (B) Validation of NASH model in HepG2 cells by ORO staining. Results are represented as mean +SD. *p<0.05, **p<0.01 and ***p<0.001 when OA is compared to Control cells.

The effect of OA on *Noct* oscillations was assessed by qPCR analysis. HepG2 cells were subjected serum shock (50 %FBS) for 2 hours to synchronize all the cells. Subsequently, cells were subjected to OA for 24 hours and then, cells were harvested for total RNA isolation every 4 hours. OA treatment caused an overall increment in *Noct* expression in HepG2 cells. There was a significant increase in *Noct* mRNA at 32 and 36 hours in OA treated cells.

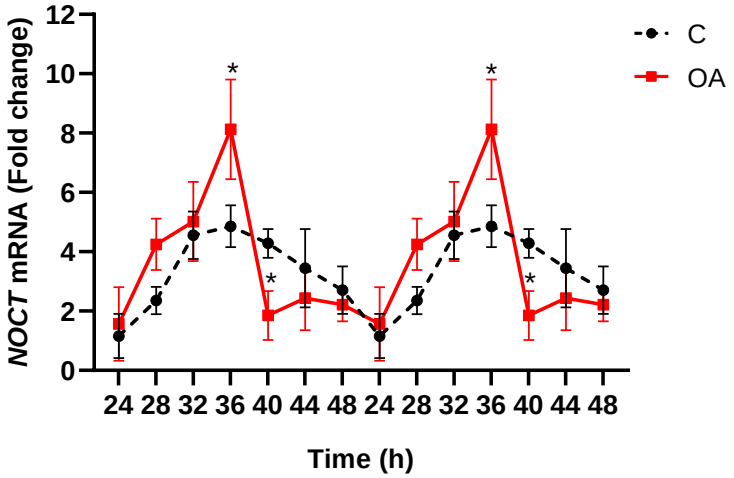
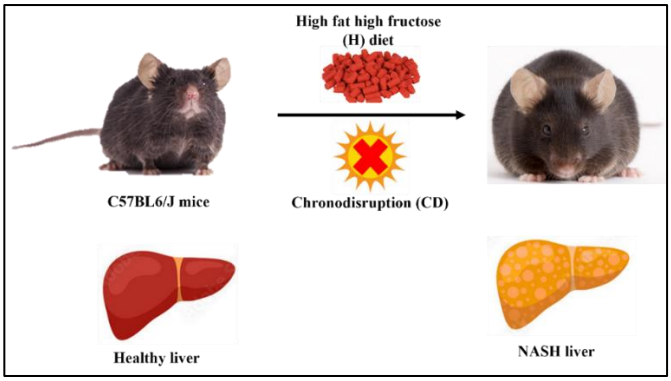


Figure 5. Alterations in oscillation pattern of *Noct* mRNA in Control (C) and OA-treated (OA) HepG2 cells. Results are represented as mean ± SD. *p<0.05, **p<0.01 and ***p<0.001 when OA is compared to Control cells.

Study 3. *In vivo* model generation of H and/or CD induced NAFLD in C57BL6/J mice and assessing hepatic Noct oscillations.



C57BL6/J male mice (6-8 weeks old) were subjected to either high fat high fructose (H) diet alone or in combination with photoperiodic shifts induced chronodisruption (CD) protocol for 16 weeks to induce NASH. Mice subjected to H and HCD showed a significant increment in body weight and had significantly elevated titres of liver function markers (ALT and AST).

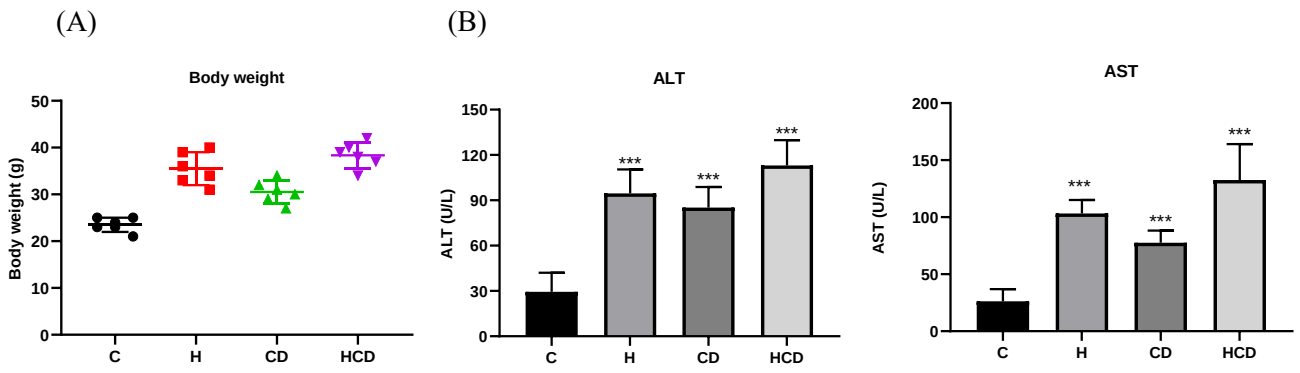


Figure 6. (A) Changes in whole body weight of C57BL/6J mice fed with high fat-high fructose (H) diet and/or subjected to chronodisruption (CD); (B) H and/or CD mediated alterations in liver function markers, AST and ALT in the serum of C57BL6/J mice. Results are expressed as mean + SD *p <0.05, and ***p <0.001 is when CD, H and HCD compared to Control (C).

The effect of H and/or CD on hepatic *Noct* expression was assessed by checking hepatic *Noct* expression at five different timepoints. H led to a significant upregulation in *Noct* mRNA at ZT12., CD caused a peak shift to ZT6 while HCD also caused a peak shift at ZT6 with a smaller peak at ZT12.

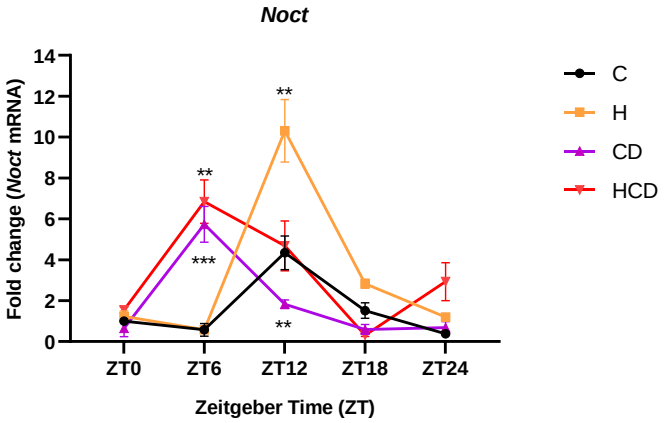


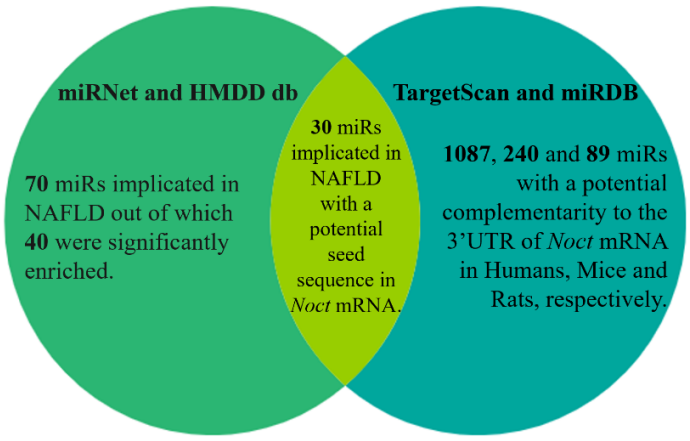
Figure 7. (A) Oscillation pattern of *Noct* mRNA in livers of mice subjected to H and/or CD regime Results are expressed as mean + SD. *p <0.05, and ***p <0.001 is when CD, H and HCD compared to Control (C).

<u>Objective 2.</u> To investigate the epigenetic regulation of *Nocturnin* in CD induced NASH.

Study 1. An *in silico* approach to investigate the role of an altered miRNA profile in regulating *Noct* expression in NASH and identifying key candidate miRNAs.

MicroRNAs (miRNAs or miRs) are non-coding RNA molecules which have important regulatory roles in hepatic metabolism and an altered miRNA expression profile is known to be a key contributing factor in the pathophysiology of liver diseases like NAFLD/ NASH. Herein, we were interested in studying whether an altered hepatic miRNA profile has a role in modulating Noct expression under the said pathology. We employed an *in silico* approach wherein, we employed miRNA databases like TargetScan and miRDB to identify all the miRNAs with a potential seed sequence in the *Noct* gene in mouse, rats and humans. We then used miRNet and Human MicroRNA Disease Database (HMDD) to identify all the miRNAs implicated in NAFLD/NASH pathology. Subsequently, we overlapped these two datasets using Cytoscape 3.9.1. software to give us clues about the potential role of an altered miRNA profile in modulating *Noct* expression in NAFLD/NASH pathology.

(A)



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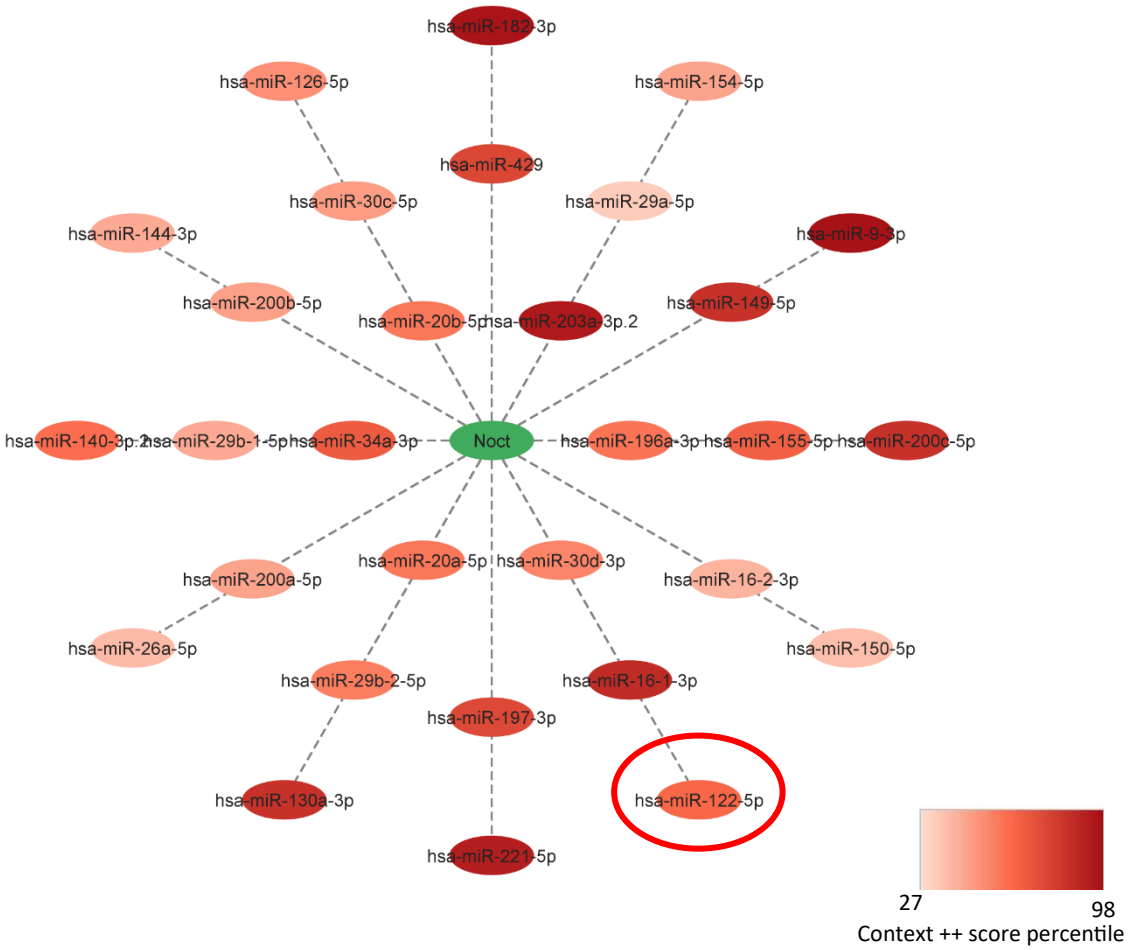


(C)

Position169-175 of Noct 3'UTR	5'	...UUGAAAAGGUGUUUGCACUCCAG...
		I I I I I I
mmu-miR-122-5p	3'	GUUUGUGGUAACAGUGUGAGGU
Position184-190 of Noct 3'UTR	5'	...UUGAGGAGGUGUUUGCACUCCAG...
		I I I I I I
rno-miR-122-5p	3'	GUUUGUGGUAACAGUGUGAGGU
Position458-464 of Noct 3'UTR	5'	...UGAAAAAGGCGUUUGCACUCCAA...
		I I I I I I
rno-miR-122-5p	3'	GUUUGUGGUAACAGUGUGAGGU

Figure 8. (A) Summary of the *in silico* study, (B) Study revealed 30 miRNAs implicated in NAFLD/NASH with a potential seed sequence in *Noct* out of which miR-122 had sufficiently high context ++ percentile scores, and (C) Further TargetScan analysis revealed that miR-122 has a conserved seed sequence in the *Noct* gene.

TargetScan and miRDB identified **1087**, **240** and **89** miRNAs with a potential seed sequence in *Noct* gene in humans, mice, and rats respectively. miRNet identified **70** miRNAs implicated in NAFLD/NASH out of which **40** were significantly enriched. Cytoscape 3.9.1 overlapped these two datasets where we came across **30** miRNAs with a potential seed sequence in *Noct* mRNA

and implicated in NAFLD/NASH, thus suggestive of the role of an altered miRNA profile in regulating *Noct* expression. MiR-122 was among these 30 miRNAs. Since miR-122 is a liver-specific miRNA having crucial regulatory roles in hepatic cholesterol and lipid metabolism, we hypothesized miR-122: Noct axis in H and/or CD induced NASH.

Study 2. Establishing association between the identified miRNA and *Noct* in H and/or CD induced NASH in *in vivo* animal model.

Most of the miRs regulate gene expression by binding to the 3'UTR of their target genes, causing transcript degradation. From our *in silico* analysis, identified miR-122 was scrutinized in the *in vivo* model of NASH that was generated in C57BL6/J male mice. We checked the hepatic miR-122 expression and correlated its expression with the hepatic *Noct* expression at ZT12. This specific timepoint was chosen since hepatic *Noct* is known to peak at ZT12. As can be seen from the graph, miR-122 levels negatively correlated with *Noct* expression: In the H group, lower miR-122 levels were observed which coincided with high *Noct* expression and the reverse was observed in the CD group. Thus, the results threw light on the possibility of miR-122: *Noct* axis functioning in the pathology.

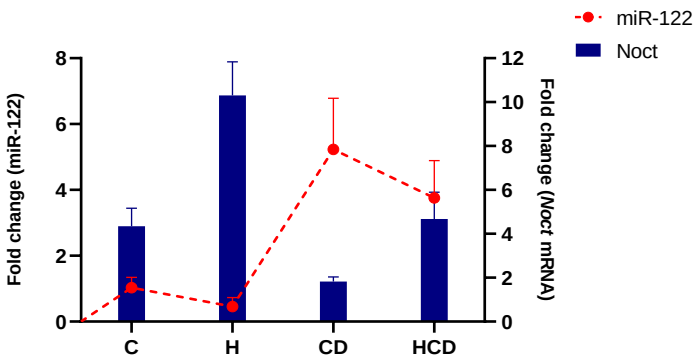
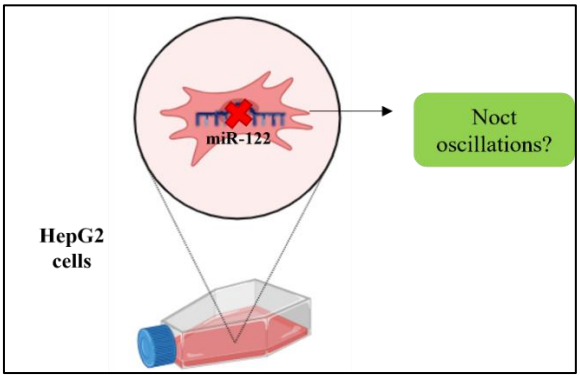


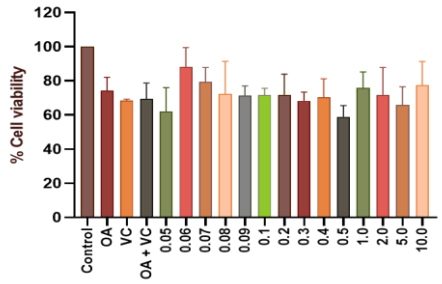
Figure 9. Correlation of hepatic miR-122 and *Noct* expression at ZT12. Results are expressed as mean + SD. *p <0.05, and ***p <0.001 is when CD, H and HCD compared to Control (C).

Study 3. Studies in HepG2 cells to validate miRNA: *Noct* association in NASH.



To further substantiate the potential miR-122: Noct axis in NASH pathology, we subjected HepG2 cells to miR-122 specific inhibitor, NSC-5476 for 24 hours. The optimum dose of this inhibitor was standardized by performing MTT assay wherein, the cells were subjected to OA alone or in combination with increasing concentrations of the inhibitor. From the results obtained, we chose 10 µM dose for all subsequent experiments. MiR-122 inhibition was validated by qPCR and further, *Noct* oscillations were checked. MiR-122 inhibition caused a significant derangement in the *Noct* oscillations with a peak shift at 36 hours and an overall increment in the *Noct* expression.

(A)



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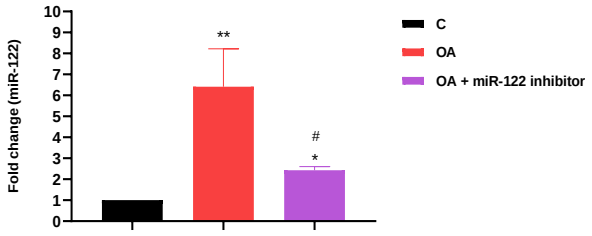


(C)

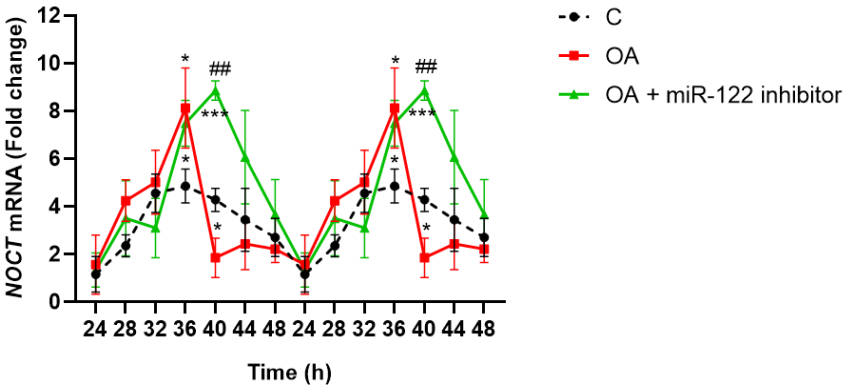


Figure 10. (A) Dose standardization of miR-122 specific inhibitor NSC-5476, by MTT Assay in HepG2 cells; (B) Validation of miR-122 inhibition in HepG2 cells by qPCR and (C) Alterations in *Noct* oscillations in HepG2 cells subjected to OA alone or in combination with miR-122 inhibitor. Results are expressed as mean + SD. *p <0.05, and ***p <0.001 is OA is compared to Control (C) and #p<0.05 when OA + miR-122 inhibitor is compared to OA.

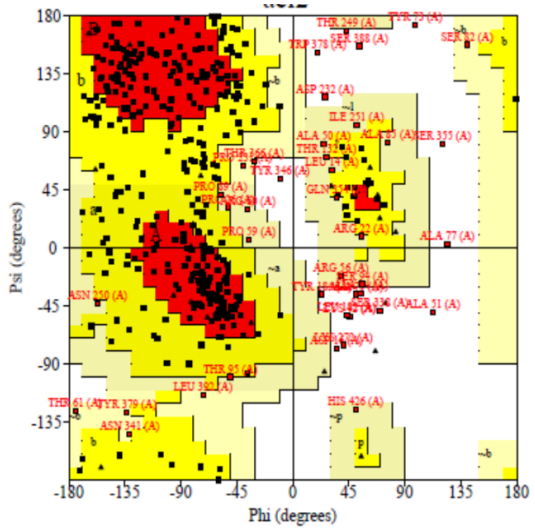
<u>Objective 3.</u> To investigate the possible melatonin vs Nocturnin synergy in H and/or CD induced NASH.

Study 1. Molecular docking studies of melatonin and NOCT.

To study any possible interaction of melatonin and NOCT, we performed molecular docking using Autodock, Pyrx and Pymol using the softwares' default parameters. The complete protein structure of mouse NOCT has not yet been deciphered. We employed iTASSER server to generate the full structure of mouse NOCT using the amino acid sequence from NCBI. The structure predictions were validated by performing Ramachandran Plot analysis in PDBSum and subsequently, we performed molecular docking. Docking results gave 9 probable docking interactions, out of which we selected the one with the highest binding energy scores and lowest RMSD values.

(A)

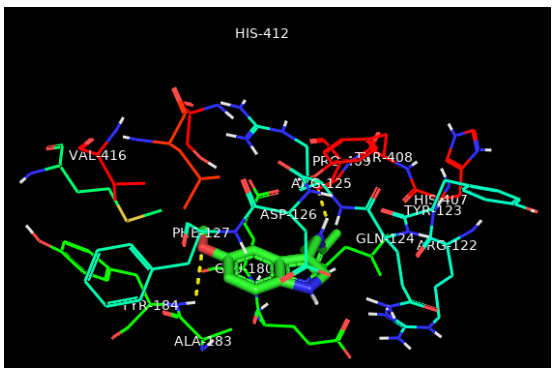
(B)



Plot statistics

Residues in most favoured regions [A,B,L]	204	54.4%
Residues in additional allowed regions [a,b,l,p]	135	36.0%
Residues in generously allowed regions [~a,~b,~l,~p]	19	5.1%
Residues in disallowed regions	17	4.5%
Number of non-glycine and non-proline residues	375	100.0%

(C)



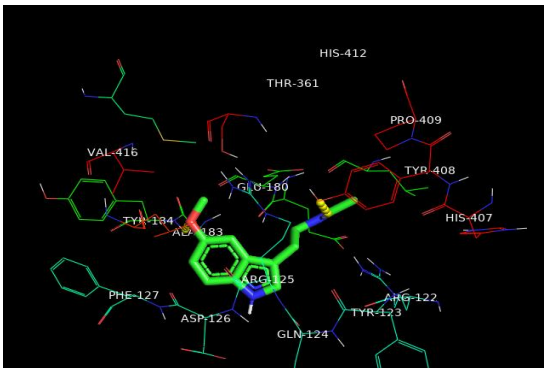
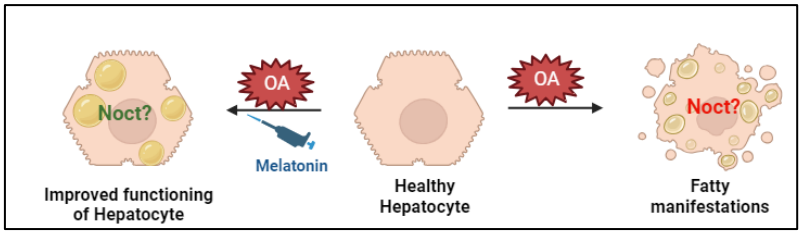


Figure 11. (A) mouse NOCT protein structure: iTASSER predictions; (B) Validation of mouse Nocturnin structure- Ramachandran plot data and (C) Model 0 was selected as the most suitable model since the interaction had the lowest RMSD values and the highest binding affinity compared to all the other models.

Study 2. Possible role of melatonin in improving Noct oscillations in HepG2 cells.



HepG2 cells were subjected to OA (0.5 mM) alone or in combination with increasing concentrations of melatonin (5µM- 1000 µM) and MTT assay was performed. From the results obtained, 100 µM concentration of melatonin was chosen for all the further experiments.

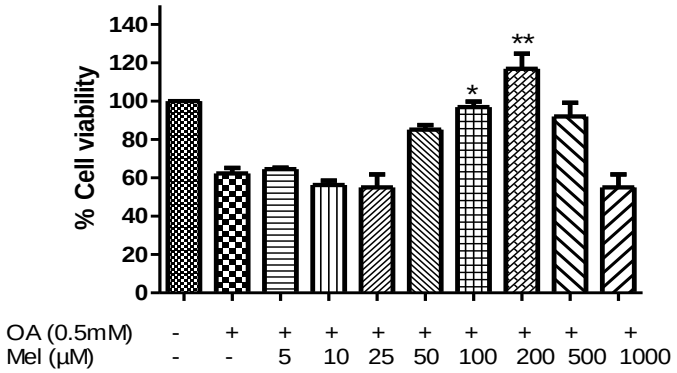


Figure 12. Graphs showing percentage cell viability (by MTT assay) after treatment with OA alone or in combination with increasing concentrations of melatonin. Results are expressed as mean + SD. *p <0.05, and ***p <0.001 when compared to Control (C).

Subsequently, possible alterations in *Noct* expression due to co-supplementation was assessed by subjecting cells to OA and/or melatonin for 24 hours and checking *Noct* expression. Melatonin treatment led to a significant decrement in *Noct* mRNA, when compared to OA treated cells.

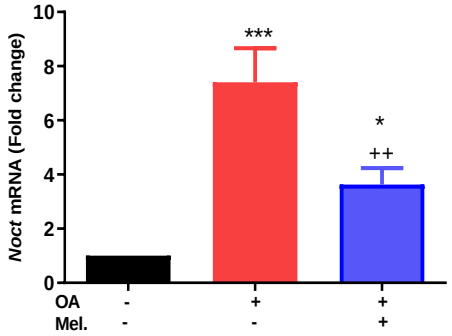
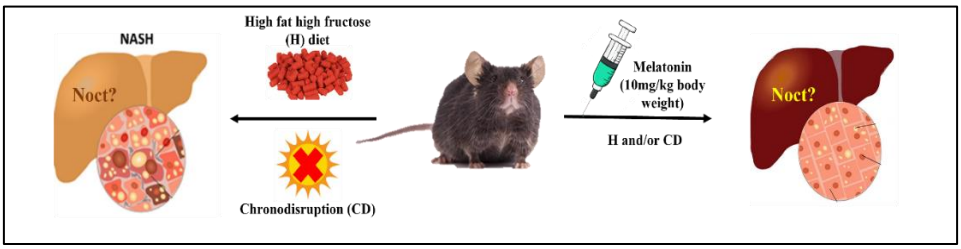


Figure 13. *Noct* expression in HepG2 cells subjected to OA alone or in combination with melatonin at ZT0. Results are expressed in as mean + S.D. *p<0.05, **p<0.01 and ***p<0.001 when OA is compared to C and ++p<0.01 when OA+ Mel is compared to OA.

Study 3. Assessing the possible role of melatonin in improving hepatic Noct oscillations in H and/or CD induced NASH pathology.



To study the hepatoprotective effect of melatonin via modulation of Noct expression, C57BL6/J mice were administered melatonin intraperitoneally (10mg/kg body weight) from the 9th week till the end of 16th week. Melatonin treatment led to decrement in the body weight when compared with H and CD groups. Melatonin treatment to HCD group however, showed no significant changes in body weight. Melatonin also led to a significant decrease in the serum liver function markers (AST and ALT), when compared to the respective disease control groups (H, CD and HCD).

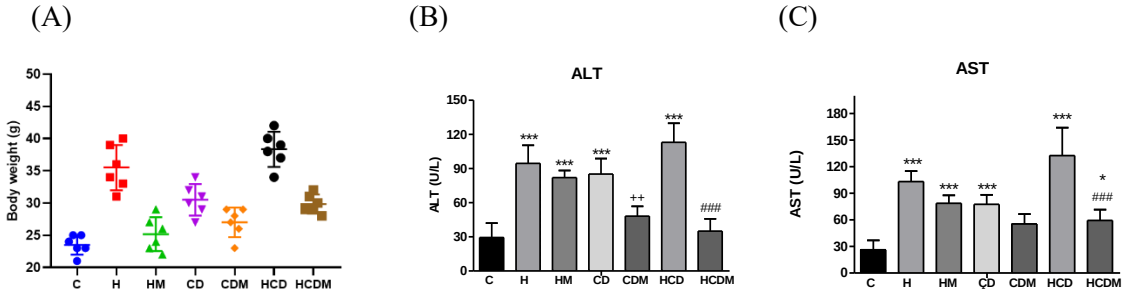


Figure 14. Changes in whole body weight of C57BL6/J mice fed with high fat-high fructose (H) diet and/or subjected to chronodisruption (CD). C57BL6/J male mice were administered melatonin from the 9th week till the end of 16 weeks. Melatonin treatment (10mg/kg/day) improved liver function as can be seen by the lowering of ALT and AST titers. Results are expressed as mean + S.E.M. *p <0.05, and ***p <0.001 is when CD, H and HCD compared to Control (C); ++p <0.01 is when CDM is compared to CD and ###p <0.001 is when HCDM is compared to HCDM.

Hepatic *Noct* mRNA was quantified at 5 time points in all the melatonin treatment groups and compared to their respective disease control groups (H,CD, HCD). Exogenous melatonin treatment showed corrective changes in the oscillation pattern of *Noct* mRNA to a great extent.

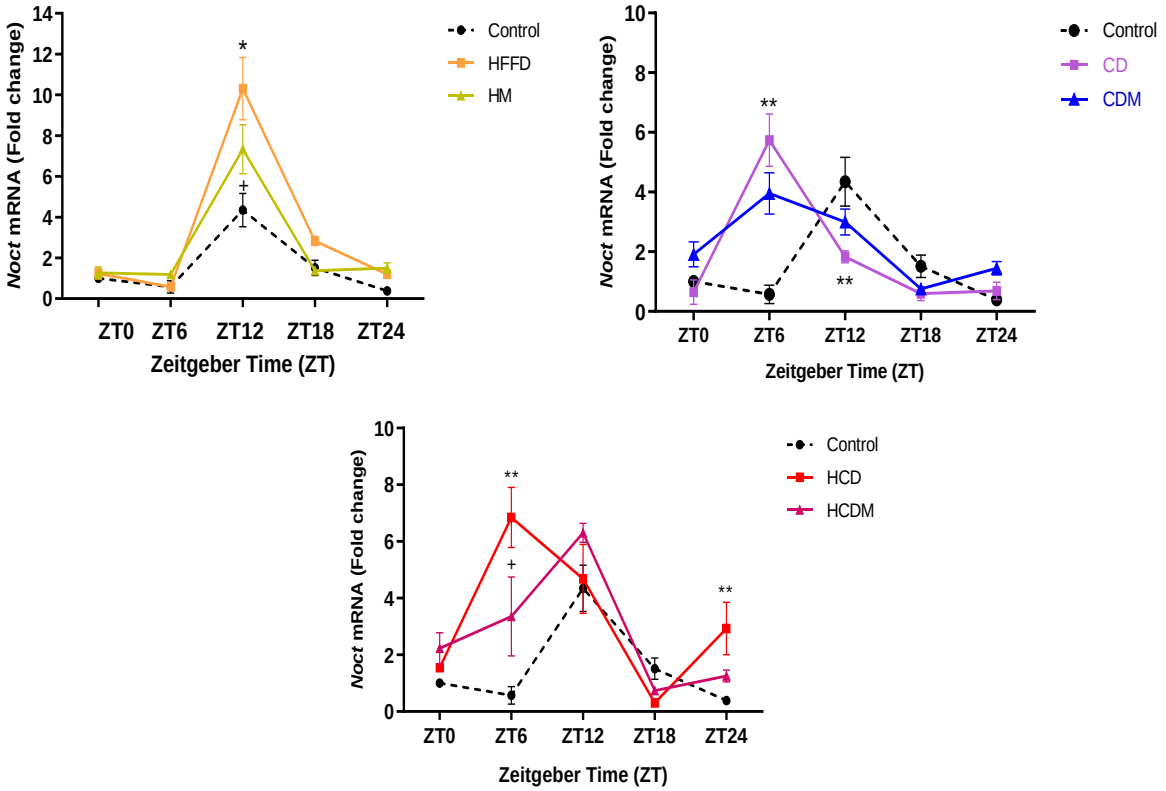


Figure 15. *Noct* mRNA at different time points. Note that melatonin treatment improved oscillation pattern of hepatic *Noct* to a great extent. Results are expressed as mean + S.D. *p <0.05, **p<0.01 and ***p <0.001 is when CD, H and HCD compared to Control (C) and +p <0.05, ++p <0.01, +++p<0.001 is when HM, CDM and HCDM is compared to H, CD and HCD respectively.

Key findings:

- Nocturnin is a recently discovered circadian output gene with important roles in regulating lipid metabolism and cytoskeleton organization.
- Photoperiodic shifts induced CD induced NASH significantly alters hepatic *Noct* oscillations (mRNA studies have been completed and Western Blotting analysis is in progress)
- Several miRNAs regulate hepatic Noct expression, out of which miR-122: Noct axis in the pathophysiology of NASH is a novel finding.
- Melatonin interacts with Noct and modulates *Noct* expression. Melatonin can correct *Noct* oscillations to a great extent in *in vivo* and *in vitro* models of NAFLD.

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