Major Liver diseases

Liver diseases are a major cause of global death indices after cancer, stroke and respiratory diseases (Sivakrishnan & Pharm, 2019). Liver diseases can be caused by infections, genetic factors, specific lifestyle conditions, excessive consumption of alcohol and shift working hours (Kulkarni et al., 2020; LIMA et al., 2019; Marchesini & Marzocchi, 2007). Chronic hepatitis B and C, alcohol and non-alcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) associated with obesity, type 2 diabetes and other diseases of metabolic syndrome forms a cluster of the most common liver diseases globally. Hepatitis B (HBV) and hepatitis C (HCV) are viral infections that impact substantial number of patients worldwide. Hepatitis C can cause serious liver damage and is known to spread through blood or body fluids of infected patients. HCV is an RNA virus of the Flaviviridae family and is diagnosed by the detection of specific antibodies and viral RNA in the serum (Hwang, 2001). On the other hand, HBV is a DNA virus of the hepadnaviridae family and is usually diagnosed by circulating hepatitis B surface antigen (HBsAg) in blood (Fattovich et al., 1991; Tagger et al., 1999). Wilson's disease, known as "progressive lenticular degenerative disease", is a combination of neurological and chronic liver diseases (Ferenci, 1998; Huster, 2010). This disease causes abnormalities in liver, nervous system and haemolytic anaemia (Ferenci, 1998). Liver cirrhosis is an end-stage liver diseases is a condition typically characterized by the replacement of normal liver tissue by scar tissue over a period of time. Initially patients with liver cirrhosis may experience fatigue, weakness, weight loss and in later stages, patients may develop severe jaundice, abdominal swelling and gastrointestinal bleeding (Du Plessis et al., 2013).

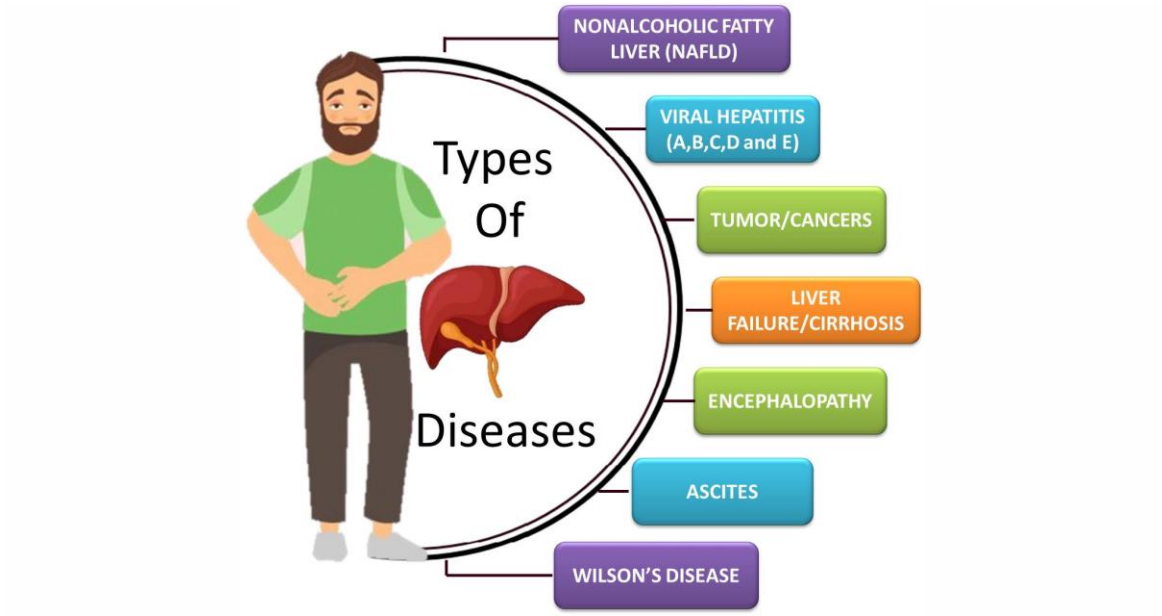

Figure 1: Types of liver diseases

Nonalcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is an umbrella term that encompasses the simple steatosis in the liver to more progressive associated hepatitis, fibrosis, cirrhosis, and in some cases hepatocellular carcinoma (Ramai et al., 2021). NAFLD is characterized by steatosis of the liver; involving more than 5% triglycerides (TG) accumulation with no evidence of hepatocyte injury whereas NASH is defined by a necro-inflammatory process whereby excess or persistent inflammation results in hepatic cell apoptosis. The pathophysiology of NAFLD is a complex multi-organ process involving metabolic and inflammatory changes in liver, adipose tissue and gut. Increased flux of fatty acids from the insulin-resistant adipose tissue or increased production of fatty acids derived from *de novo lipogenesis* (DNL) result in increased hepatic triglyceride (TG) synthesis and accumulation (Arab et al., 2018). Liver steatosis can mount metabolic stress due to development of lipotoxicity, mitochondrial dysfunction, reactive oxygen species (ROS) production, immune cells migration and production of pro-inflammatory cytokines (Peters et al., 2018). NAFLD progresses towards nonalcoholic steatohepatitis (NASH) that is triggered by pro-inflammatory signals from the adipose tissue inflammasomes or by bacterial products such as endotoxin coming from the leaky gut (Ray, 2015). The metabolic and inflammatory changes associated with NASH can lead to hepatocyte necrosis, tissue damage and subsequently varying degree

of scar tissue formation (i.e. liver fibrosis). In a subset of affected individuals, NASH can ultimately progress to an end-stage disease comprising of liver cirrhosis and hepatocellular carcinoma (HCC) (Anstee et al., 2013; Jahn et al., 2019).

NAFLD is one of the important causes of liver diseases that are the consequential effect of global pandemic of metabolic syndrome. Global prevalence of NAFLD was about 15% in 2005 which had increased to 25% in 2010. In recent times, the cases of NASH have doubled from 33% to 59.1% (Lonardo et al., 2016). Patient studies from India have revealed that obesity, sedentary lifestyle, unhealthy dietary pattern and metabolic syndrome are the major contributors for NAFLD with a higher incidence rate amongst obese and diabetic patients (Kalra et al., 2013). With lack of direct therapies available for NASH, it is now considered as the second most common condition requiring liver transplantation (Anstee et al., 2013).

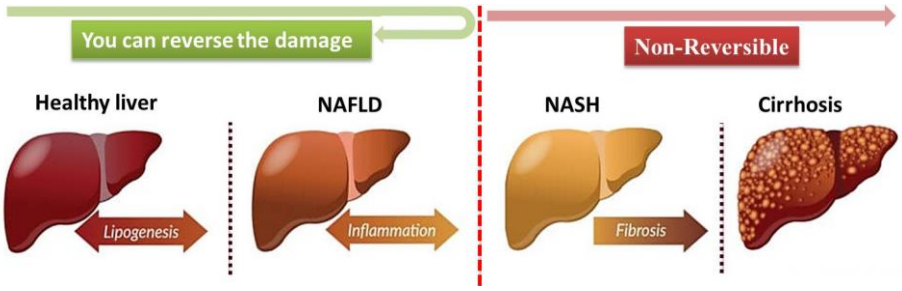

Figure 2: Reversible and non-reversible stages of nonalcoholic fatty liver disease

Risk factors for the development of NAFLD:

- Consumption of Western diet (high calorie diet)
- Metabolically obese with lean phenotype.
- Genetic causes: PNPLA3 variants; congenital defects of metabolism like familial hypobetalipoproteinaemia, lysosomal acid lipase deficiency etc.
- Endocrine disorders such as excess glucocorticoids, Polycystic Ovarian Syndrome (PCOS), hypothyroidism, male hypogonadism or deficiency in growth hormone.
- Drug related causes, effective by amiodarone, methotrexate or tamoxifen.

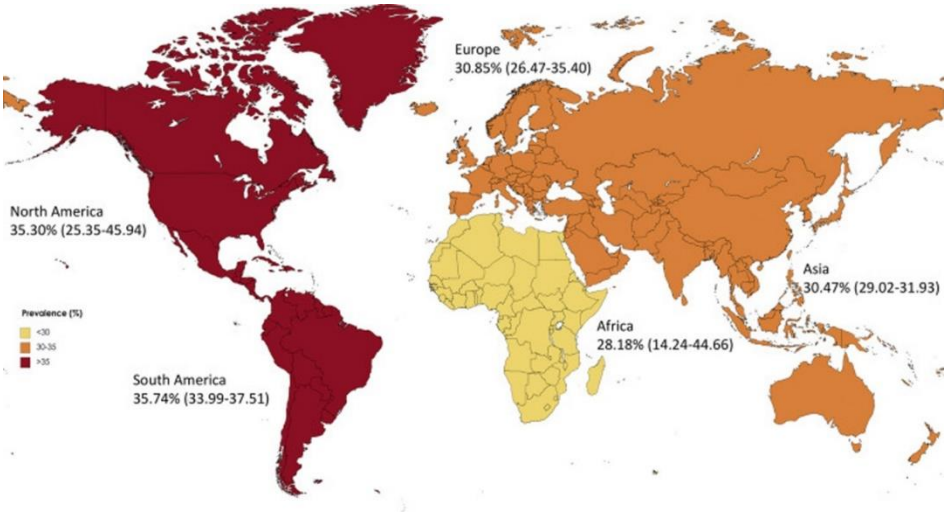

Figure 3: Continent-wise prevalence of NAFLD in the year 2019 (Le et al., 2021).

Non-Alcoholic Steatohepatitis (NASH)

Ludwig first coined the term "Non-alcoholic Steatohepatitis (NASH)" in 1980 describing the pathophysiological condition of fatty liver in non-alcoholic individuals (Ludwig et al., 1980). The pathophysiology of NASH is complex and multifactorial with multiple systemic alterations in liver (Parthasarathy et al., 2020). The 'two hits' theory consists of a first 'hit' with intrahepatic accumulation of fatty acids, and a second 'hit' including other factors such as oxidative stress, mitochondrial dysfunction, apoptosis and gut-derived lipopolysaccharides (LPS) (Day & James, 1998). The diversified characteristics of NAFLD is reflected in patients with development of isolated steatosis of the liver, whereas the progressive stage termed as non-alcoholic steatohepatitis (NASH) has typical characters such as ballooning, inflammation, hepatocyte injury, and resulting in fibrosis (Parthasarathy et al., 2020). Asymptomatic nature of NASH is one of the major reasons for diagnosis, treatment and scaling prevalence lacks clarity (Armstrong et al., 2012). Since the last two decades, there exists a persistent increment in the number of individuals being diagnosed with NASH in Asian countries. People suffering from obesity, insulin resistance (IR) type 2 diabetes mellitus, and dyslipidemia are the most important risk factors and are at high risks of getting NASH (Amarapurkar et al., 2007). In general, the risk of NAFLD increases with the degree of obesity and it is associated with increased visceral fat and insulin resistance, however, NAFLD risk is also modified by other physiological attributes, such as the extent of peripheral versus visceral adiposity, and the degree of insulin resistance in adipose depots and other insulin-sensitive tissues, such as muscle and liver (Jun et al., 2008; S. H. Park et al., 2007; Vernon et al., 2011). Thus, body size [e.g. body mass index (BMI)] per se is an imperfect predictor of NAFLD risk, and NAFLD can also occur in the context of a normal or low BMI. The severity of hepatic damage (i.e., NASH) generally correlates with the degree of hepatic metabolic stress, but there is significant inter-individual variability regarding NASH outcomes.

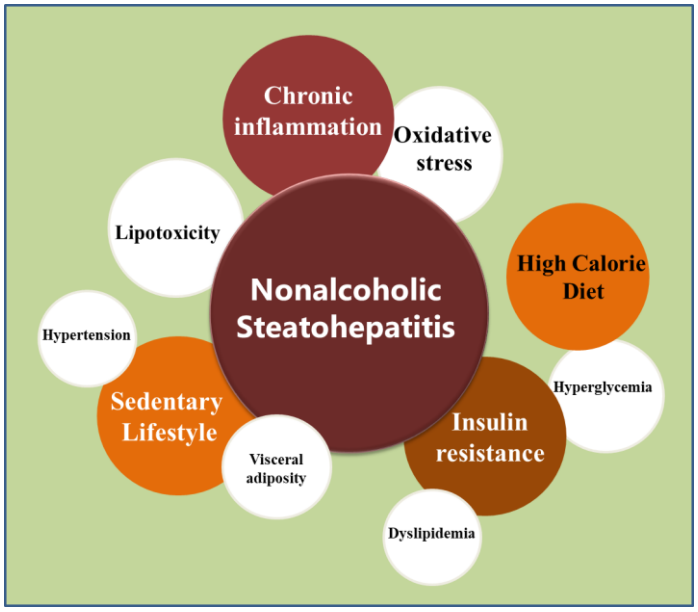

Figure 4: Association of metabolic syndrome components with nonalcoholic steatohepatitis

Lipotoxicity, along with activation of the innate immune system are the major drivers of NASH. Lipid-induced sub-lethal and lethal stress culminates in the initiation/activation of pro-inflammatory processes involving Kupffer cells, monocytes, neutrophils, macrophages, dendritic cells and lymphocytes that contribute to the progression of the inflammatory cascade (Y. Chen & Tian, 2020). Further, the contribution of other immune cell types, such as neutrophils and B cells, is an area of intense research (Alkhouri et al., 2009, 2012; Farrell et al., 2018). Many factors, such as dysfunction in mucosal barrier, leaky gut, intestinal inflammation, increased bacterial translocation in liver through gut–liver axis modifies an individual's susceptibility to NASH (Parthasarathy et al., 2020). In rodent experimental models, high fructose intake has been associated with increased plasma glucose, triglycerides, dyslipidaemia, hepatic gluconeogenesis, endoplasmic reticulum (ER) stress, hepatocellular ballooning, inflammation, increased systolic blood pressure, impaired insulin signalling along with progression towards liver fibrosis (Ter Horst & Serlie, 2017).

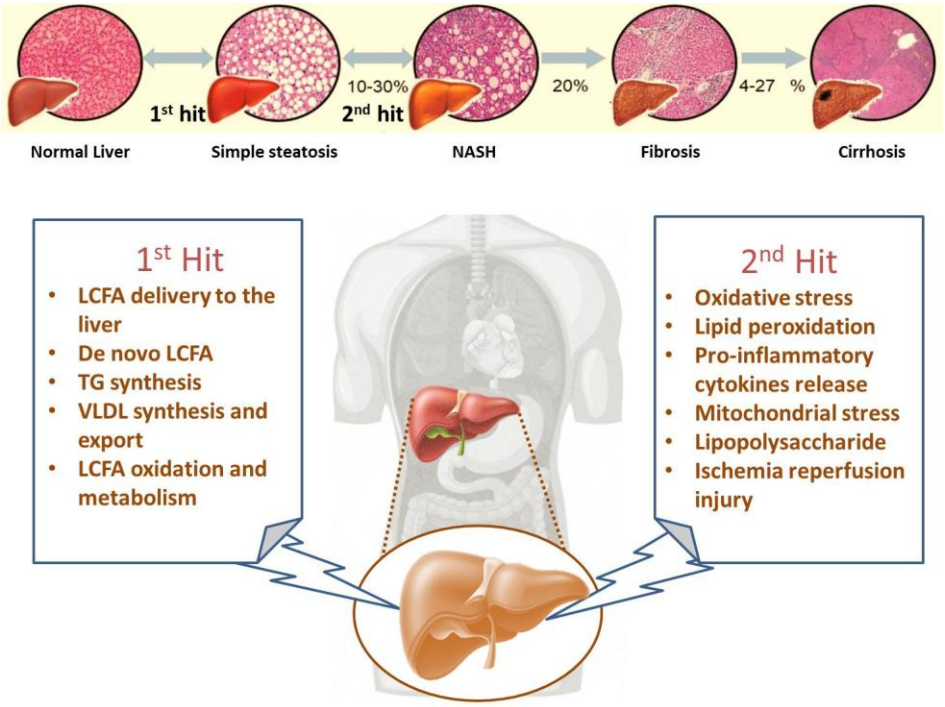

Figure 5: The "two-hit" hypothesis in the progression of Nonalcoholic fatty liver disease (NAFLD) to Nonalcoholic steatohepatitis (NASH)

Gut–brain axis

Preliminary postnatal existence in mammals represents a phase of bacterial colonization. In humans, the lower intestine contains 10^{14}–10^{15} bacteria, that is 10–100 times more bacteria in the gut than eukaryotic cells in the human body (10^{13}) (Thursby & Juge, 2017). The microbiome is a dynamic system, influenced by several factors, including stress, genetics, unbalanced diet, metabolism, age, geography, and antibiotic treatment. The two most abundant phyla are Firmicutes and Bacteroidetes accounts for at least 90–95% of the gut microbiome along with minor phyla such as Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia that are present in reduced numbers (Eckburg et al., 2005; Mahowald et al., 2009). Recent studies show that bacteria, including commensal, probiotic, and pathogenic bacteria in the gastrointestinal (GI) tract can activate neural pathways and central nervous system (CNS) signalling and shows how microbiota are important in normal healthy brain function (Ma et al., 2019). The bi-directional conveyance between the gut and central nervous system has long been documented. Several pathways of this conveyance include the neuroendocrine system,

the autonomic nervous system (ANS), the enteric nervous system (ENS), and the immune system in gut-brain axis (Carabotti et al., 2015).

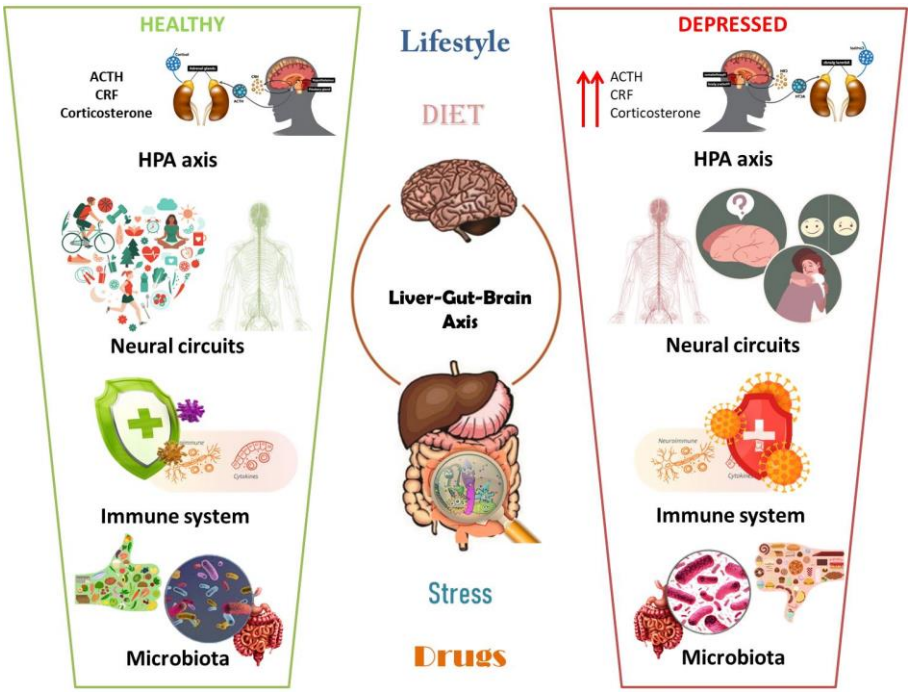

Figure 6: The Liver-gut-brain axis.

In depressed patients, a direct link between microbiota and dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis with exaggerated cortisol has been reported (Knorr et al., 2010; Mohajeri et al., 2018). In addition to modulating HPA axis function, microbiota may influence CNS function directly through neuronal, hormonal, humeral and immunological activation. Stress is known to increase intestinal permeability, thus allowing bacterial antigens and LPS to translocate across the intestinal mucosa and directly access both immune cells and neuronal cells of the ENS (Bieghs & Trautwein, 2014). In models of diet-induced obesity and in genetically modified obese (*ob/ob*) mice, administration of a broad-spectrum antibiotic prevented metabolic syndrome by improving glucose tolerance, reduced weight gain and fat mass and lowered adipose inflammatory markers (Cani et al., 2008; Hyogo et al., 2002). Using a mouse model of chronic anxiety and depression via olfactory bulbectomy, investigators showed that elevated corticotrophin-releasing hormone (CRH) expression, increased c-Fos activity,

serotonin levels, and colon motility were associated with an altered intestinal microbiome, which was suggested to be due to the activation of the HPA axis (A. J. Park et al., 2013). Thus, the altered microbiota profile and high corticosterone levels results in behavioral shifts in mice or rats subjected to high calorie diet.

Hepatic-hippocampal crosstalk

Depression and Anxiety

Depression and anxiety are commonly seen in patients suffering from NAFLD, NASH, Fibrosis and end stage liver disease such as hepatic encephalopathy and Cirrhosis (Choi et al., 2021; LIMA et al., 2019). Obesity is associated with the risk of antidepressant resistance in patients suffering from major depression as compared to control subjects with normal weight. Alterations in gut microbiota associated with obesity are in conjunction with elevated inflammatory cytokines (IL-1β, IL-6, TNFα and NfκB) and CRP levels which is also observed in patients with depression and anxiety-like behavior (Dinan, 2009; Jantaratnotai et al., 2017; Roberts et al., 2003; Rothermundt et al., 2001). In an animal model of obesity and depression induced by high-fat diet (HFD) combined with chronic unpredictable mild stress (CUMS), elevated levels of IL-1β, IL-6, TNF-α, NF-κB and microglial activation were reported in hippocampus and prefrontal cortex in disease control groups signifying depression-like behavior (Wang et al., 2022). Under conditions of chronic HFD feeding, changes in neurotrophic signaling were observed which culminates into elevated NFκB transcription, pro-inflammatory cytokine expression, antigen-presenting markers and reactive gliosis in nucleus accumbens resulting into anxiety-like behavior in C57BL/6 mice (Décarie-Spain et al., 2018). Upon HFD feeding, increased levels of pCREB was recorded in nucleus accumbens producing conditions of anhedonia and behavioral despair in rodents (Barra de la Tremblaye, 2016; Mi et al., 2017). Long term administration of high fat diet results into neuro-inflammation, depression and anxiety-like behavior along with changes in inflammatory responses due to alterations in Nf-κB, CCL2, PPARβ and PPARγ in prefrontal cortex; however no memory impairment was observed due to long lasting obesity in male rats (Lorena et al., 2021). Consumption of high fat diet has been shown to decrease in

exploration of novel object, disruption of intracellular cascades involved in synaptic plasticity, increased corticosterone levels, upregulation of inflammatory cytokines, activation of innate immune system and altered insulin/glucose homeostasis in rats which can be due to anhedonia or increased anxiety (Dutheil et al., 2016; Glushchak et al., 2021; Kosari et al., 2012).

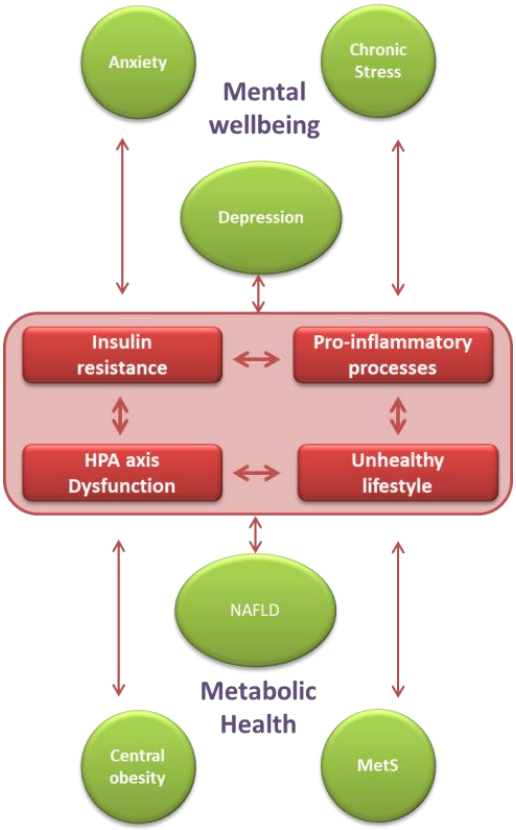

Figure 7: The liver-brain crosstalk in the condition of lifestyle disorder.

The cyclic AMP acts as a key messenger in several G-protein coupled receptor signaling pathways regulating cell growth and differentiation as well as in gene and protein expression. Likewise, cAMP signaling appears to play a key role in the pathophysiology of depression. In depression, signaling via cAMP may be impaired by cyclic nucleotide phosphodiesterases (PDEs), with PDE4 gene family playing a major role in regulating cognition and depressive disorders (Wong et al., 2006; Y. Xu et al., 2011). High fat diet fed rats showed increased activity of PDE4 especially in hypothalamus which can alter cAMP/PKA signalling pathway and may cause depression

(Vagena et al., 2019). It is worth noting that anxiety related behaviour is only induced during the time-window of prolonged HFD ingestion. The involvement of disturbed gut microbiota in the aetiology of depression is supported by the observation that transplantation of gut microbiota from patient with depression into gut of germ-free mice or anti-biotic treated mice and rats leads to depression like phenotypes (L. Chang et al., 2022; Cooke et al., 2021). The abundance of Bacteroidetes is reduced and Firmicutes were increased in faecal samples upon administration of high calorie diet which is also observed in patients with depression (Magne et al., 2020; Stojanov et al., 2020; Wu et al., 2018). In humans and experimental models, the Firmicutes to Bacteroidetes ration increased with increase in depressive symptoms, suggesting a positive correlation between them (J.-J. Chen et al., 2020; Koliada et al., 2017; Y. Liu et al., 2021; Magne et al., 2020). In addition to that, HFD weakens the endothelial tight junctions in the intestine which induces neuro-inflammation in brain (De Aquino et al., 2019; Nguyen et al., 2020). Neuroinflammation also seems to play an important role in the establishment of an anxiety-like phenotype and depression, where higher levels of TNFα and Mcp-1 have been reported in the offspring of rodents fed HFD during pregnancy and lactation (White et al., 2009). It has been seen that a prolonged state of inflammation, especially by TNF-α, leads to activation of immune cells, producing a toxic neuro-environment, and promoting neuronal loss, which disrupts neuro-behavior (Butterworth, 2013; Guillemot-Legris & Muccioli, 2017; Moreno-Navarrete et al., 2017). In humans as well as in rodent experimental models significant findings suggest that stress/HFD-induced alterations increase corticosterone (CORT), neuronal atrophy, low-grade inflammation while decreasing in hippocampal neurogenesis and neurotrophic factor expressions in brain (Arcego et al., 2018; Del Rosario et al., 2012; Vagena et al., 2019).

Spatial learning and memory

Spatial learning and memory refers to an individual's ability to navigate and form episodic memories. In brain, hippocampus and medial entorhinal cortex are the keys areas governing spatial learning and memory (Pilly & Grossberg, 2012). Conditions like cognitive dysfunction, gut dysbiosis, impaired urea cycle function and systemic ammonia accumulation are frequently linked to metabolic diseases such as NAFLD. Such alterations in crosstalk of liver-gut-brain axis are imperative in pathogenesis of

neuropsychiatric syndrome like hepatic encephalopathy which is associated with progressive liver disease. Lower discrimination indices, exploration time in novel object recognition test along with alterations in hippocampal GABA, glutamate and glutamine concentrations were recorded in mice fed with high fat-high fructose diets in C57BL/6 mice (Martínez-Orozco et al., 2021). Animals subjected to high calorie diets shows significant memory impairment and pathological changes in hippocampus with reduced nerve spines in CA1 and impaired long-term potentiation (Alzoubi et al., 2018; Gladding et al., 2018; Klein et al., 2016).

Cytokines plays an important role in promoting mood deficit and diet induced obesity as sustained elevations can elicit region-specific inflammation and non-homeostatic alterations in neuroplasticity. High-fat feeding triggers activation of microglia, increase in apoptosis of neuronal progenitor cells, increased expression of hippocampal pro-inflammatory markers that elicit depression-like behaviour, reduces adult hippocampal neurogenesis with impaired functions (Underwood & Thompson, 2016). Alterations in the function of the hippocampus are also suggested by studies that showed decreased expression levels of genes important for learning and memory, decreased expression of brain growth factors and increased neuro-inflammation, although the direction of these changes varied and may thus depend on the specific type and length of dietary exposure (Fulton et al., 2022).

Circadian rhythms

Biological rhythms are also called circadian clock and refer to behavioral, physiological and molecular changes in an organism. Biological rhythms are the repetitive natural cycle of change in organism's body chemicals or functions. The internal master clock controls and coordinates other peripheral clocks in our body. There are different types of biological rhythms such as circadian rhythm (approximately 24-hour cycle or one day), ultradian (rhythm with shorter period than 24 h), infradian (rhythm that last more than 24 h) and circannual rhythms (rhythm that last 1 year).

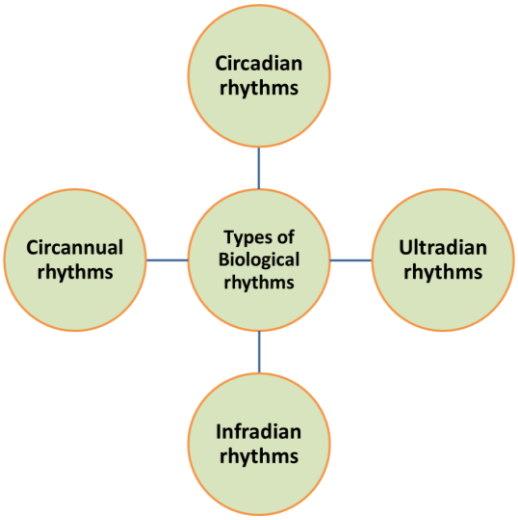

Figure 8: Types of Biological rhythms

Everyday circadian clock needs to be reset by various environmental cues, referred to as 'Zeitgeber' (German for "time giver") (Dubruille & Emery, 2008). Sun light is considered as the most strongest, reliable and predictable Zeitgeber for the entrainment of the circadian clock of an individual. Light has been regarded as a main regulator of biological system and affect circadian clock gene expression in various non-SCN clocks like the pineal gland, adrenal gland, liver, pancreas through autonomic innervation. The self-sustained, autonomic circadian oscillators are present in peripheral organs such as liver, intestine, heart and retina contributes to metabolic processes via clock genes in these organs (Dibner et al., 2010). The suprachiasmatic nucleus (SCN) functions as the master circadian pacemaker defining the various diurnal/nocturnal activities in animals and comprises of three major components viz. input pathway, core clock and the output pathway (Gekakis et al., 1998). The core circadian clock forms the rhythms according to

external cues and relays the rhythmic signals via output pathway to other peripheral organs for various physiological activities through neuronal and endocrine pathways such as sympathetic nervous system, glucocorticoid signalling and feeding-fasting behavior (Welsh et al., 2010). Although light has been traditionally considered as the key regulator and the main Zeitgeber for the circadian system, the time of food intake and the sleep-wake cycle has a strong impact on liver, pancreas, kidney, intestines and heart clock without affecting the main circadian clock in the SCN (Damiola et al., 2000). Early studies have identified that bilateral lesions of the SCN in the rodents result in complete loss of rhythmic locomotor activity, body temperature, food consumption, drinking behavior and hormone release (la Fleur et al., 2001). Many experimental studies have revealed the relationship between circadian rhythms, physiology and metabolic diseases (Joshi et al., 2021; Shirsath et al., 2021). Hence it becomes imperative to study the role of altered circadian rhythms in diseases like NAFLD, NASH, atherosclerosis and auto-immune disorders.

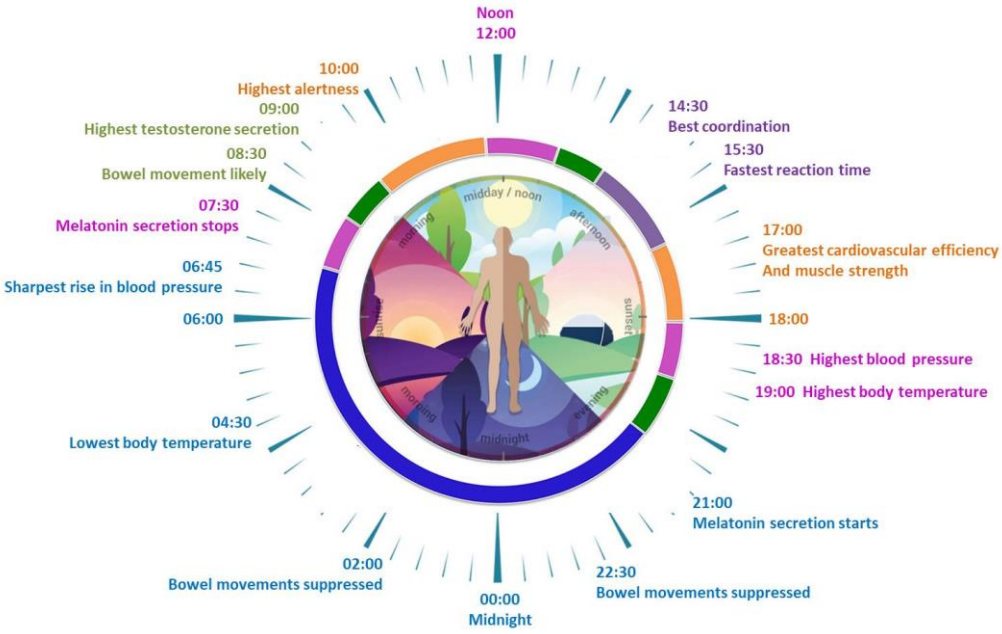

Figure 9: Circadian rhythms in daily physiological functions.

Circadian clock genes

The circadian molecular machinery consists of the transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein (BMAL1), which heterodimerize in the cytoplasm and migrate into the nucleus to activate the expression of many other clock-controlled genes (Okamura et al., 2002; Reppert & Weaver, 2002). CLOCK/BMAL1 drives the expression of PERs (Per1, Per2) and CRYs (Cry1, Cry2), that heterodimerize and translocate from the cytoplasm to the nucleus to inhibit the core molecular clock genes CLOCK/BMAL1 (Kwon et al., 2006). When PER and CRY levels are low, CLOCK/BMAL1 reinitiate their transcription. The CLOCK/BMAL1 dimers also initiate the transcription of second feedback loop which acts in coordination with PERs and CRYs (Kwon et al., 2006). This involves the transcription of the orphan nuclear-receptor genes Rev-Erbα/β and RORα/β. These orphan nuclear receptor genes then compete for Retinoic acid-related Orphan receptor response element (RORE) binding sites within the promoter of BMAL1 where ROR proteins induce Bmal1 transcription and REV-ERB protein inhibits the same (Zhao et al., 2020). Thus, CLOCK/BMAL1 drives the expression of Rev-erbs and RORs, which in turn regulate the rhythmic expression of Bmal1. In adult animals, a double knockout of Rev-Erbα and β was found to be lethal during development and thus revealed that the Rev-erbs are essential for normal Period gene regulation of circadian behavioural rhythmicity (Ikeda et al., 2019).

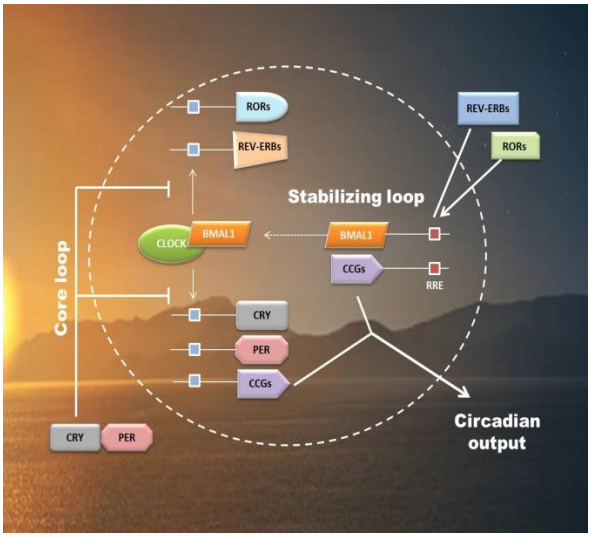

Figure 10: Molecular mechanism of circadian clock

Hepatic circadian clock

The SCN driven peripheral clocks in the liver performs many crucial roles in maintaining liver homeostasis, including energy metabolism, expression of enzymes, lipid & bile acid metabolism, immunity, xenobiotic detoxification and storage of the nutrients (Kondratov et al., 2006; Marcheva et al., 2010; Tahara & Shibata, 2016). Feeding time is an important regulator of the circadian clock in peripheral tissues like liver. Disruption or alterations in feeding hours accelerates the development of various liver diseases such as NAFLD, cirrhosis, hepatitis and liver cancer (Adamovich et al., 2014; Bugge et al., 2012; Moro et al., 2016; Mteyrek et al., 2016; Yamajuku et al., 2009). Several studies have indicated that individual cell types in liver have their autonomous circadian oscillators and regulate important physiological roles (Kondratov et al., 2006; Marcheva et al., 2010; Sadacca et al., 2011). Hepatic transcriptome analysis showed circadian variation in the expression of many genes related to oxidative metabolism, gluconeogenesis, lipogenesis, bile acid synthesis, mitochondrial biogenesis and amino acid turnover suggesting that hepatic circadian clocks control and regulate these genes (Bass & Takahashi, 2010; Lamia et al., 2008, 2009). Disruption or mutations in the mice Clock gene ($Clock^{\Delta 19/\Delta 19}$) showed decreased circulating insulin, increased cholesterol, glucose, leptin and TG levels as compared with wild-type control mice (Bass & Takahashi, 2010; Doi et al., 2010; Marcheva et al., 2010). Cryptochromes (Cry1 and Cry2) play a pivotal role in maintenance of circadian rhythms by acting as transcription repressors and are also important for the control of energy metabolism (E. E. Zhang et al., 2010). The clock proteins CRYs are essential players in the regulation of the IGF-1 signalling and hepatic glucose metabolism (Chaudhari et al., 2017). Thus the role of CLOCK/BMAL1 and other clock genes are essential in maintaining the liver circadian clock.

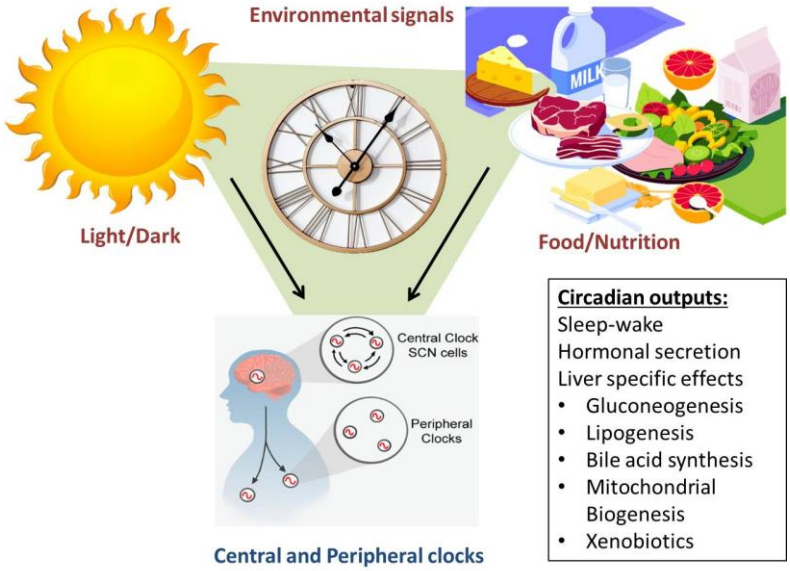

Figure 11: Circadian clock mediated control and coordination of metabolic functions of liver

In hepatocytes, Bmal1 ablation reduced the expression of the glucose transporter 2 (Glut2) leading to decreased post-absorptive glucose uptake in mice (Lamia et al., 2008). PGC-1α, the master regulator of mitochondrial biogenesis and also the primary regulator of liver gluconeogenesis is rhythmically expressed in the liver and skeletal tissues of mice (C. Liu et al., 2007). PGC-1α stimulates the expression of Bmal1 and Rev-erbα through the activation of orphan nuclear receptors of the ROR family. Mice lacking PGC-1alpha showed abnormal diurnal activity rhythms, body temperature, metabolic rate along with the aberrant expression of circadian clock genes and energy metabolism (C. Liu et al., 2007).

Clock-mutant mice had obesity and increased levels of cholesterol and TG. Thus, multiple genetic studies in mouse models had established the critical role of core circadian clock and other genes as a critical regulator of glucose, lipid and bile acid metabolism in mouse liver (Pan et al., 2020; Turek et al., 2005). Increased circulatory levels of free fatty acids (FFAs), TG and cholesterol were found in hepatocyte-specific ablation in Rev-Erbα/β (Bugge et al., 2012). Thus circadian clock controls lipid metabolic enzymes. Oscillatory patterns of hepatic triglycerides synthesis genes such as Glycerol-3-Phosphate Acyltransferase 2 (Gpat2), 1-acylglycerol-3-phosphate O-acyltransferase1/2 (Agpat1/2), Lipin1/2 and Diacylglycerol O-acyltransferase 2 (Dgat2)

that regulate TG synthesis from glycerol-3-phosphate was found circadian in nature (Adamovich et al., 2014). The circadian clock gene Rev-erbα controlled the expression of Insig2 and SREBP1C, the key metabolic genes of lipogenesis (Le Martelot et al., 2009). Bile acids (BAs) also act as signalling molecules and are physiological ligands for Farnesoid X receptor (FXR) and G-protein coupled receptor (GPBAR1) or TGR5 can activate signalling molecules involved in mitogen-activated protein kinase (MAPK) pathway (Goodwin et al., 2000; Guo et al., 2016; Watanabe et al., 2011). Bile acids synthesis is controlled by transcriptional feedback loop of FXR, SHP and FGF15 in mice (Goodwin et al., 2000). The expression of FXR and SHP60 in liver and the secretion of FGF15 in intestine is controlled by CLOCK (Stroeve et al., 2010). Both FXR and SHP60 drive the rhythmic expression of Cyp7a1, the rate limiting enzyme in the conversion of cholesterol to bile acids (Björkhem & Eggertsen, 2001; Stroeve et al., 2010). DBP (D-Box Binding PAR BZIP Transcription Factor) is a protein exhibiting a robust rhythm in suprachiasmatic nuclei and transcription of DBP is activated by heterodimerization of CLOCK-BMAL1 through E-boxes and inhibited by Per and Cry proteins (Yamaguchi et al., 2000). DBP plays an important role in cholesterol homeostasis through circadian transcription regulation of Cyp7a1 (Lavery & Schibler, 1993). In Rev-erbα deficient mice, hepatic expression of E4BP4, small heterodimer partner (SHP), both negatively regulate expression of CYP7A1 (Duez et al., 2008). Thus, REV-ERBα is in the feedback loop of bile acid synthesis by regulating hepatic CYP7A1, SHP and E4BP4.

Circadian genes not only control lipid metabolites but also the regulation of lipid biosynthesis. The circadian pattern of cholesterol synthesis was achieved through the oscillatory expression of (HMG-CoA reductase) and CYP7A1 (Gnocchi et al., 2015; Xing et al., 2020). In rodent models, bile acid profile follows a circadian rhythmicity in serum, liver, gallbladder and intestines (Ferrell & Chiang, 2015). In clock-mutant mice arrhythmic expression of clock-genes (Bmal1 and Per2), as well as the lipid metabolic genes such as HMG-CoA reductase, LDLr and Cyp7a1 displayed altered circadian rhythms (Gnocchi et al., 2015). C57BL/6J mice fed with high-fat high fructose diet for 16 weeks showed an altered circadian pattern of expression of clock and clock-controlled genes (CLOCK, BMAL1, PER1, PER2 and CRY2) in the liver as compared to control (Joshi et al., 2021). A study from our lab had shown that, in high-fat high fructose diet fed C57BL/6J mice, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC),

SREBP1c, and fatty acid-binding protein 4 (FABP4) showed an altered expression in liver and the same was altered in chronodisruption group as well (Joshi et al., 2021).

Circadian clock and Nonalcoholic fatty liver disease (NAFLD)/Nonalcoholic steatohepatitis (NASH)

Shift works causing alterations in the expression of circadian genes are the basis of inflammatory liver diseases like NAFLD and NASH (James et al., 2017; Shimizu et al., 2018). In transition from NAFLD to NASH, several factors participate such as mitochondrial dysfunction, ER stress, oxidative stress, inflammation, insulin resistance etc. Besides controlling systemic metabolism, the circadian clock machinery plays a critical role in regulating autophagy, ER stress and oxidative stress (Farrell et al., 2018; Hashimoto et al., 2015; Ludwig et al., 1997). The hepatic circadian oscillator maintains the daily pattern of food availability by expressing a large number of genes regulating metabolism and physiology (Adamovich et al., 2014; Akhtar et al., 2002). Mainly, sleep-wake and feeding- fasting cycle regulates the circadian rhythms. Knockout of circadian clock gene Bmal1 in mice produces severe phenotypes such as arrhythmic sleep-wake cycles, lean body, reduced longevity and irregular energy metabolism as compared to wild-type mice (Kondratov et al., 2006; Marcheva et al., 2010). Studies showed that Cry deficient mice have dwarfism and double Cry1/Cry2 mice knockout show disrupted IGF-1 rhythms, abnormal triglycerides levels in serum and liver, elevated corticosterone levels along with glucose intolerance as compared to wild type mice (Chaudhari et al., 2017). High fat diet fed $Clock^{\Delta 19/\Delta 19}$ mice exhibited increased weight gain, body fat accumulation, lipid and glycogen accumulation in hepatocytes as compared with high-fat diet fed control mice (Turek et al., 2005). B6xC57 mice subjected to western diet and sleep disruption for 6 h/day for 5 days, significantly suppressed core clock genes, altered Cyp7a1, increased circulating cholesterol and free fatty acids as compared to control (Ferrell & Chiang, 2015). Thus, short term circadian disruption also majorly impacts the hepatic clock gene expression, bile acid and lipid metabolism (Shi et al., 2019).

Circadian rhythm and gut microbiota in NAFLD

The gut microbiota provides fermentation of non-digestible substrates like dietary fibres, endogenous intestinal mucus and supports the growth of special microbes that produces major short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (Valdes et al., 2018). Besides liver-restricted functions and processes, extrahepatic tissues also play a crucial role in NAFLD and its progression to NASH. Obesity-associated alterations in the gut microbiota (i.e., dysbiosis), increased endotoxins and alterations in the Firmicutes: Bacteroidetes composition and their interactions with the host (intestinal epithelial cells) has been implicated as an aetiological agent in the pathogenesis and progression of NAFLD to NASH (Abu-Shanab & Quigley, 2010; Ahmed et al., 2020; C.-J. Chang et al., 2015; Magne et al., 2020). In turn, microbiota-derived lipopolysaccharide (LPS) can perturb hepatic lipid metabolism and increase hepatic inflammation by modulating the production of short-chain fatty acids and altering the BA pool composition, which may influence intestinal and hepatic FXR activity thereby altering BA metabolism, thus affecting both glucose and lipid homeostasis (Boyer et al., 2006; Sinal et al., 2000; Watanabe et al., 2011). Circadian perturbations (i.e. mutation of core circadian clock-components or jet lag) lead to dysbiosis and development of metabolic pathologies. Furthermore, mutations in innate immune genes (Tlr5, Nlrp6 and Nlrp3) play pivotal roles in sensing gut microbiota, hence modulating metabolic pathologies including NAFLD (Zheng et al., 2020).

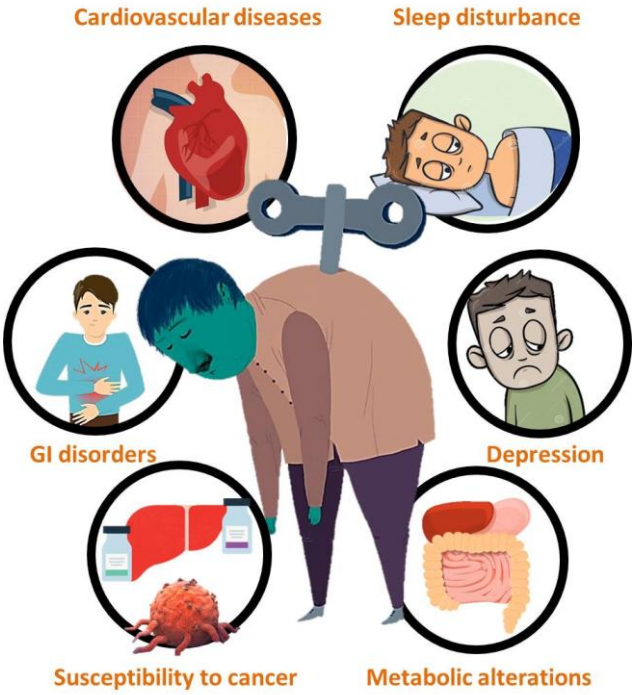

Figure 12: Chronodisruption mediated perturbations in psychiatric and metabolic pathologies

Circadian clock and Neuro-behavioral anomalies

Sleep timings are regulated by the circadian system but sleep is a complex biological process that is also regulated by homeostatic mechanisms (Patel et al., 2021). Evidences suggest that discrepancies in circadian rhythm and sleep wake cycle are important in pathophysiology of mood disorders. Patients showing symptoms of depression often suffer from sleep disturbances, diurnal mood variations and altered circadian rhythms (Germain & Kupfer, 2008; Lam, 2008). Thus, sleep-wakefulness cycle disruptions have been associated with Major Depressive Disorder (MDD) in patients. Studies indicated that Rapid eye movement (REM) sleep has an important role in dreaming, memory, emotional processing and healthy brain development and the same gets affected under conditions of depression and anxiety (Genzel et al., 2015; Maquet et al., 1996; Modell & Lauer, 2007). Studies have also shown an arrhythmic circadian pattern of cortisol, thyroid-stimulating hormone (TSH), melatonin and heart rate rhythms in depressed individuals (Dienes et al., 2013; Vadnie & McClung, 2017). In major

depressive disorders (MDD), the circadian genes (CRY1, CLOCK and REV-ERBα) have been implicated in several recent studies (Bunney et al., 2015; Lamont et al., 2022).

In Zebra finches, artificial light at night (ALAN) impairs learning, memory and suppresses nocturnal melatonin levels and neurogenesis (Moaraf et al., 2020). In chronodisrupted rat model induced by artificial light at night, age dependent redox insults and neurochemical deficits were reported wherein, a significant increment in ROS, lipid hydroperoxidation, protein carbonyl, nitric oxide was observed while total thiol, ferric reducing antioxidant potential level, superoxide dismutase and catalase activities were reduced in the brain of ALAN exposed groups with higher amplitude in aged rat (Verma et al., 2022). In NAFLD patients, sleep fragmentation can worsen the metabolic disease especially with obstructive sleep apnea and is imperative in promoting the progression of NAFLD to NASH through persistent oxidative stress and inflammation (Parikh et al., 2019).

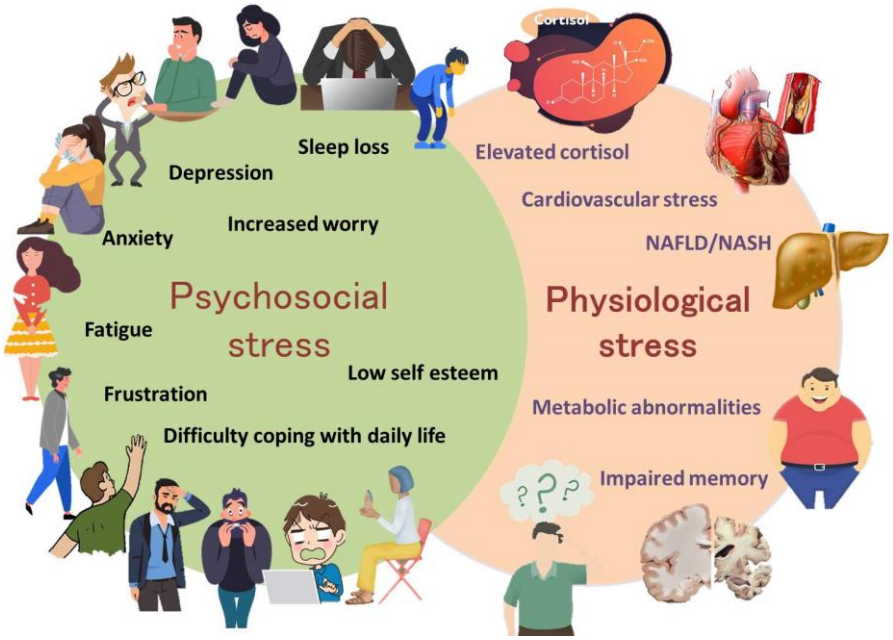

Figure 13: Alterations in neuro-behavior due to psychosocial and physiological stress

Melatonin and Lifestyle disease

Melatonin is a hormone (N-acetyl-5methoxytryptamine) produced especially at night from the pineal gland (located in brain) along with harderian gland, gut-mucosa, cerebellum, airway epithelium, liver, kidney, adrenals, pancreas, thyroid gland and

thymus (Kvetnoy et al., 2001; R. Reiter et al., 1999). Several other cells such as bone marrow, lymphocytes, epithelial cells, retina have also been reported to secrete melatonin and follow a biological rhythm (D. Tan et al., 2003). Melatonin synthesized in other organs seems to be used locally as a paracoid, autocoid, tissue factor and as an antioxidant (D. Tan et al., 2003). The suprachiasmatic nuclei (SCN) of the hypothalamus has melatonin receptors and melatonin has a direct action on SCN to influence circadian rhythms (Weaver et al., 1993). Melatonin is metabolized to 6- hydroxy-mel in the liver and the main metabolite excreted is 6-sulphatoxy-mei. Depending on the concentrations, melatonin acts as a hormone, receptor independent autocrine and paracrine antioxidant, direct radical scavenger, immune-modulator, anti-aging factor and as an anti-carcinogen (Guerrero & Reiter, 2002; Gurunathan et al., 2021; Konturek et al., 1997; D.-X. Tan et al., 2015). The most basic function of the indole is speculated to be its antioxidant actions by detoxifying reactive oxygen species (ROS) including hydrogen peroxide (H2O2) , hydroxyl radical ('OH), peroxyl radicals (ROO-) and also reactive nitrogen species (RNS) such as nitric oxide radical (NO-) and peroxinitrite (ONOO) (Hardeland, 2017; R. Reiter et al., 1999; D.-X. Tan et al., 2015; D. Tan et al., 2003).

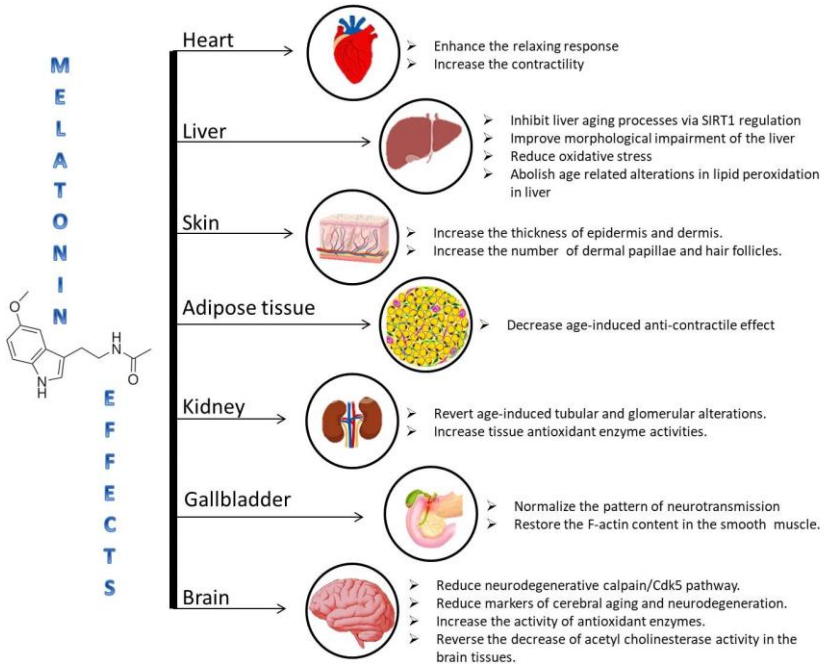

Figure 14: Protective role of melatonin on various organs

The function of melatonin in the gut in regulating intestinal mobility, the immune system, GI secretion and released of peptide involved in energy metabolism remains unclear (Aydin et al., 2008). Melatonin is involved in regulating chloride (Cl^-) secretion in colon, in turn controlling the intestinal ion transport (Chan et al., 1998; Cuzzocrea et al., 2001). In different physiological conditions, melatonin is known to protect the colon by modulating the anti-oxidant mechanism against stress-induced and ischemia-induced lesions (Konturek et al., 1997; R. J. Reiter, 2003). In high fat diet fed mice, oral melatonin supplementation improves lipid accumulation and is associated with reprogramming of the gut microbiota, especially the abundance of Bacteroidetes and alistipes-mediated acetic acid production thus alleviating intestinal dysbiosis (Yin et al., 2018). Exogenous melatonin treatment shows significant decrease in liver steatosis, expression of inflammatory cytokines (TNF-α, IL-1β, and IL-6) and reduced phosphorylation of P38 and JNK1/2 in HFD fed mice (Sun et al., 2016). Further melatonin treatment (50 mg/kg) for one month was able to alleviates methionine-and choline-deficient diet induced NASH by decreasing oxidative stress, pro-inflammatory cytokines levels, liver steatosis and hepatocyte apoptosis (Tahan et al., 2009). Melatonin was able to improve CCL4 induced liver fibrosis via corrective changes in the expression of BMAL1, CLOCK, PER1, PER2, CRY1 , CRY2 and retinoic acid receptor-related orphan receptor (RORα) (González-Fernández et al., 2018). Thus, melatonin prevents metabolic syndrome diseases such as obesity, NAFLD, inflammatory bowel diseases etc. by improving liver inflammatory status, circadian clock genes oscillations and modulating the gut microbiota in various experimental models of lifestyle disorder.

Melatonin & Neurodegenerative diseases

Melatonin holds many chronobiotic and cytoprotective properties. Melatonin as a cytoprotective molecule reverses the low degree inflammatory damage in various neurodegenerative disorders and aging. In elderly Alzheimer's disease (AD) patients, significant low levels of melatonin were reported as compared to normal people at the same age (Nous et al., 2021). Melatonin inhibits the aggregation of amyloid-beta protein into beta-sheets which is the most commonly implicated marker of Alzheimer’s disease (Poeggeler et al., 2001). Melatonin also reduces the hyper-phosphorylation of tau protein, which leads to the neurofibrillary tangles of Alzheimer's disease (WANG & WANG,

2006). In experimental models of Alzheimer's disease and Parkinson's disease (PD), melatonin improves low-grade neuro-inflammation by inhibiting amyloid-beta aggregation into beta-sheets and hyperphosphorylation of tau-protein (Antolín et al., 2002; He et al., 2010; Permpoonputtana et al., 2018; Vincent, 2018; WANG & WANG, 2006).

Brain-derived neurotrophic factor (BDNF) a growth factor and a member of neurotrophin family primarily binds to its receptor, tropomyosin receptor kinase B (TrkB) and their combination results in neuronal survival, growth, differentiation, and synaptic plasticity (Bramham & Messaoudi, 2005; Rattiner et al., 2004; Yoshii & Constantine-Paton, 2010). The correlations between stress or anti-depressant is based largely on up- or downregulation of BDNF (Lee & Kim, 2010; Martinowich et al., 2007). Various studies had postulated that reduced levels of BDNF leads to atrophy and cell loss in hippocampus and prefrontal cortex in depressed subjects (Banasr et al., 2011; Erickson et al., 2012; Youssef et al., 2018). Rodent models subjected to high-fat diet induced oxidative stress and neuro-inflammation in the hippocampus showed depression and impaired spatial learning and memory (Alzoubi et al., 2018; Arcego et al., 2016, 2018; Vagena et al., 2019; J. Xu et al., 2019). Melatonin treatment is known to improve cognitive impairment by reducing oxidative stress, neuro-inflammation and increasing the BDNF expression in hippocampus of rats subjected to sleep deprivation (L. Zhang et al., 2013). Anti-depressant potential of melatonin by an improvement in BDNF and TrkB levels in hippocampus of fluoxetine treated male C57BL/6N mice endorses the potential of melatonin in bringing about corrective changes (Li et al., 2018). Recent studies had shown that exogenous melatonin can enhance cognitive performance through neuroprotective mechanism against HFD induced neurobehavioral stress (Alzoubi et al., 2018; Ramírez-Rodríguez et al., 2009). Oral administration of melatonin can improve antioxidant status and neuroprotective effects against consumption of HFD in Wistar rats (Alzoubi et al., 2018). Thus, melatonin can be a potent neuroprotective agent improving cognitive, depression, anxiety as well as neuro-inflammatory mechanisms leading neuro-behavioral perturbations in lifestyle disorder.

Crux of the study

Chronodisruption in combination with high fat-high fructose diet represent an ideal model of lifestyle disorder that has been employed in this study. Individual effects of high fat-high fructose diet or chronodisruption have also been studied and data has been put to scrutiny through a series of molecular biology experiments as well as the neuro-behavioral studies in the rodents. The study makes a profound investigation on whether chronodisruption in combination with high fat-high fructose diet, is able to cause changes in the development/onset and progression of nonalcoholic steatohepatitis (NASH). Further, the crux is to study hepatic-hippocampal crosstalk, wherein; the gut microbiota and the bile acid metabolism genes have been studied so as to establish a meaningful correlation between the changes taking place in liver function, bile acids and gut microbiota vis-à-vis the neuronal functions. Since bile acids are known to cross the blood-brain barrier, the tauro- and urso-deoxycholic acids have been widely implicated in the brain functions and modulation of behavior. In the final phase of the study, BDNF-TrkB pathway in the hippocampus and the behavioral experiments establish the efficacy of melatonin mediated improvement in behavioral patterns and reparative changes in BDNF-TrkB pathway. The novelty of the study is the experimental model of lifestyle disorder that investigates changes in behavioral patterns viz. locomotor deficits, anxiety, depression, spatial learning and memory followed by melatonin mediated corrective changes. Overall, the proposed objectives of the hepatic-hippocampal crosstalk have been achieved through a series of carefully scripted protocols.