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Hepatocellular Carcinoma in Mice Affects Neuronal Activity and Glia Cells in the Suprachiasmatic Nucleus

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My Mother and sisters

Especially for my beloved father in heaven

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Zusammenfassung

Das hepatozelluläre Karzinom (HCC) ist der häufigste maligne Lebertumor und stellt weltweit die dritthäufigste Ursache für krebsbedingte Todesfälle dar. HCC verursacht entzündliche Veränderungen sowie eine fibrotische Umstrukturierung der Leber und führt letztlich zu einer Organfunktionsstörung. Patientinnen und Patienten mit HCC leiden häufig unter anhaltender chronischer Erschöpfung, die als krebsassoziierte Fatigue (cancer-related fatigue, CRF) bezeichnet wird. CRF ist bislang nicht ausreichend erforscht; jedoch spielen vermutlich krebsassoziierte inflammatorische Prozesse, metabolische Veränderungen und Schlafstörungen eine wesentliche Rolle. Bei den Schlafstörungen handelt es sich vermutlich um Veränderungen im zirkadianen System.

Bei Säugetieren stellt der Nucleus suprachiasmaticus (SCN) des Hypothalamus eine Kernkomponente des zirkadianen Systems dar. Er erzeugt zirkadiane Rhythmen von Physiologie und Verhalten und sorgt für ihre Synchronisation mit dem 24-stündigen Hell-Dunkel-Zyklus der Umwelt, ein Vorgang der als Entrainment bezeichnet wird. Neuron-Glia-Interaktionen im SCN tragen wesentlich zur funktionellen Integrität des zirkadianen Systems bei. Diese Interaktionen sind unter verschiedenen pathologischen Bedingungen anfällig für inflammatorische Reaktionen und/oder oxidativen Stress, was zu einer Beeinträchtigung von zirkadianer Rhythmogenese und Entrainment führen kann. In der aktuellen Forschung gerät der Einfluss peripherer Pathologien, einschließlich Lebererkrankungen, auf das Gehirn im Rahmen der Leber-Hirn-Achse zunehmend in den Fokus. Über die spezifischen Auswirkungen der HCC auf das zirkadiane System ist bislang jedoch wenig bekannt.

Ziel der vorliegenden Studie war es daher, den Einfluss des HCC auf den zentralen zirkadianen Schrittmacher im SCN zu untersuchen. HCC wurde chemisch in adulten männlichen Mäusen induziert. Gruppen von HCC-tragenden sowie Kontrollmäusen wurden zu vier unterschiedlichen Tageszeitpunkten getötet. Mittels Immunfluoreszenz wurden die essenziellen SCN-Neuropeptide vasoaktives intestinales Peptid (VIP) und Arginin-Vasopressin (AVP) analysiert. Weiterhin wurden mittels Immunhistochemie Marker für das molekulare Uhrwerk (Bmal1), neuronale Aktivität (c-Fos), Astrozyten (glial fibrilläres saures Protein, GFAP), Mikroglia (ionized calcium-binding adapter molecule 1, IBA1) und für oxidativen Stress (8-Hydroxy-2′-Desoxyguanosin, 8-OHdG) im SCN untersucht.

Unsere Ergebnisse zeigen eine erhöhte Immunreaktivität von VIP, AVP, Bmal1, GFAP, IBA1 und 8-OHdG bei HCC-tragenden Mäusen im Vergleich zu Kontrolltieren,

insbesondere während der Aktivitäts- bzw. Dunkelphase. Demgegenüber war die c-Fos-Expression bei HCC-tragenden Mäusen vermindert, insbesondere während der späten inaktiven Phase.

Zusammenfassend weisen unsere Befunde darauf hin, dass HCC das zirkadiane System auf Ebene des SCN beeinflusst. Dies umfasst Veränderungen der Neuropeptidexpression, des molekularen Uhrwerks, der neuronalen und glialen Aktivität sowie eine erhöhte oxidative Belastung im SCN der Maus.

Summary

Hepatocellular carcinoma (HCC) is the most common malignant liver tumor and represents the third leading cause of cancer-related mortality worldwide. HCC induces inflammatory alterations and fibrotic remodelling of the liver, ultimately resulting in organ dysfunction. Patients with HCC frequently suffer from persistent chronic exhaustion, referred to as cancer-related fatigue (CRF). Although CRF remains insufficiently understood, cancer-associated inflammatory processes, metabolic alterations, and sleep disturbances are thought to play a major role. These sleep disturbances are presumably associated with alterations of the circadian system.

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus constitutes a main component of the circadian system. It generates circadian rhythms of physiology and behaviour and synchronizes them with the 24-hour environmental light–dark cycle, a process known as entrainment. Neuron–glia interactions within the SCN are essential for the functional integrity of the circadian system. Under various pathological conditions, these interactions are susceptible to inflammatory responses and/or oxidative stress, which may impair circadian rhythm generation and entrainment. In recent research, the influence of peripheral pathologies, including liver diseases, on the brain within the framework of the liver–brain axis has gained increasing attention. However, little is currently known about the specific effects of HCC on the circadian system.

The aim of the present study was therefore to investigate the impact of HCC on the central circadian pacemaker in the SCN. HCC was chemically induced in adult male mice. Groups of HCC-bearing and control mice were sacrificed at four different time points of the day. Immunofluorescence was used to analyse the essential SCN neuropeptides vasoactive intestinal peptide (VIP) and arginine vasopressin (AVP). In addition, immunohistochemistry was performed to assess markers of the molecular clock (Bmal1), neuronal activity (c-Fos), astrocytes (glial fibrillary acidic protein, GFAP), microglia (ionized calcium-binding adapter molecule 1, IBA1), and oxidative stress (8-hydroxy-2′-deoxyguanosine, 8-OHdG) in the SCN.

Our results demonstrate increased immunoreactivity of VIP, AVP, Bmal1, GFAP, IBA1, and 8-OHdG in HCC-bearing mice compared with control animals, particularly during the active (dark) phase. In contrast, c-Fos expression was reduced in HCC-bearing mice, especially during the late inactive phase.

In summary, our findings indicate that HCC affects the circadian system at the level of the SCN. This includes alterations in neuropeptide expression, the molecular clock, neuronal and glial activity, as well as increased oxidative stress within the murine SCN.

Hepatocellular Carcinoma in Mice Affects Neuronal Activity and Glia Cells in the Suprachiasmatic Nucleus

List of abbreviations

5-FU	5-Fluoruracil
8-OHdG	8-hydroxy-2′ -deoxyguanosine
A.U.	Arbitrary Units
ACE	Angiotensin-Converting Enzyme
AFP	Alpha Feto Protein
AMP	Adenosine-monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AVP	Arginin- vasopressin peptide
BCLC	Barcelona Clinic Liver Cancer
BDL	Bile duct ligation
Bmal1	Brain and muscle ARNT like 1
cAMP	cyclic adenosine monophosphate
CCG	Clock controlled genes
CLIP	Cancer of the Liver Italian Program
Clock	Circadian locomotor output cycles Kaput
CREB	cyclic AMP response element-binding protein
CRF	Cancer related fatigue
CRH	Corticotropin-Releasing Hormone
Cry	Cryptochrom
CT	calretinin
CT	Computertomography
DEN	Diethylnitrosamine
DNA	Desoxynucleotid Acid
e.g.	For example
ERK	Extracellular signal-related kinase
et al.	Et alli/alliae
GABA	Gamma-aminobutyric acid

GFAP	Glial Fibrillary acidic protein
GnRH	Gonadotropin-Releasing Hormone
GRP	gastrin releasing peptide
GY	Gray
H2O	water
H2O2	Hydrogen peroxide
HBV/HCV	Hepatitis B Virus/ Hepatitis C Virus
HCC	Hepatocellular carcinoma
HPA	Hypothalamus-pituitary-adrenal
IBA1	Ionized-calcium binding adapter molecule 1
IDO	Indoleamine 2,3-dioxygenase
IF	Immunoflourescence phosphate-buffered saline
igG	Immunoglobuline G, Antibody
IGL	intergeniculate leaflet
IHC	Immunohistochemistry
IL	Interleukin
ipRGC	intrinsic photosensitive retinal ganglion cells
IR	Immunoreaction
IR Induced	Irradiation induced
KCL	Potassium chlorid
LUC	Luciferin
MAPK	Mitogen-activated protein Kinase
MOA	Median of activity
MRI	Magnetic resonance Imaging
NaCL	Sodium chloride
NAFLD	non-alcoholic fatty liver disease
NMDA	N-Methyl-D-Aspartate
NO	Nitrite oxide
NPC	Neuronal precursor/stem cells
NPY	Neuropeptide Y

NT	neurotensin
p-	Phosphorylated
PACAP	Pituitary adenylyl cyclase activating polypeptide
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Triton X-100
Per	Period
p-ERK	Phosphorylated-ERK
PFA	Paraphormaldehyde
pTMN	Pathological staging screening
PVN	Paraventricular nucleus
RFA	Radiofrequenceablatioon
RHT	Retinohypothalamic tract
RILD	Radiotherapy-induced liver disease
RNA	Ribonuleic acid
Rora	RAR-related orphan receptor alpha
SCN	Nucleus suprachiasmaticus
TACE	Trans arterial chemoembolization
TNF-a	Tumor Necrosis Factor alpha
TRH	thyrotropin-releasing hormone
VIP	Vasoactive intestinal Peptide
ZT	Zeitgeber Time

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1 Introduction

1.1 Hepatocellular carcinoma

HCC is the most common primary hepatic tumor that accounts for 8% of cancers (Swain & Jones, 2019) leading to about 1 million deaths annually worldwide with higher prevalence in Asia than in the Western world. HCC is approximately twice as common in males (Altekruse et al., 2009) and liver cirrhosis represents the greatest risk factor, for which alcohol is the most common cause, followed by chronic viral hepatitis and non-alcoholic fatty liver disease (NAFLD) (Ott et al., 2012). Liver cirrhosis leads to chronic remodelling and regeneration processes, resulting in the development of genetic mutation of liver cells (Ghouri et al., 2017)(Asafo-Agyei & Samant, 2023; Da et al., 2022). Toxins such as aflatoxin, are also considered to be a risk factor for the development of HCC (Tümen et al., 2022). Lifestyle is strongly correlated with risk of HCC development e.g. lack of physical activity, obesity and smoking (Zelber-Sag i et al., 2021). Furthermore, chronodisruption and sleep disturbances via influencing the immune system, resilience to oxidative stress, and metabolic functions, could increase the risk of HCC development (Qian et al., 2025).

Absence of specific symptoms and lack of diagnostic markers represent major challenge for early diagnosis (Ren et al., 2020). The typical symptom of HCC includes right upper abdominal pain and weight loss; however, many HCC patients remain asymptomatic. The radiological investigations such as sonography, computer tomography (CT) or magnetic resonance imaging (MRI) are important diagnostic tools for the detection of HCC and the levels of alpha-fetoprotein (AFP) may not be specific enough as a diagnostic criterion as it increases in other inflammatory diseases such as chronic viral hepatitis and, thus, it should only be used for treatment monitoring (Takashima et al., 1982).

For HCC pathological and clinical staging, there are different classifications such as the pTMN classification, as well as the classification of the Cancer of the Liver Italian Program (CLIP) or the Barcelona Clinic Liver Cancer (BCLC). In addition, Okuda classification includes liver function in the staging criteria, which is throughout relevant to the patient's prognosis (Befeler & Di Bisceglie, 2002).

HCC is considered as a therapy-resistant tumor (Ren et al., 2020). The surgical options for HCC include liver transplantation as a curative treatment due to complete removal of the tumorous cells. However, the limited number of donors represents a challenge, in addition to the contraindications, for example, metastasis to other organs (Jazmati et al., 2023). In patients without cirrhosis and with intact liver function and excluded vascular invasion, partial liver resection is also a possible therapy. Small HCC tumors with difficult-accessible location can be ablated via ethanol injection or by radiofrequency ablation (RFA), which has been gained significant importance in recent years. Furthermore, RFA can also serve as a bridging period until a donor for a liver transplant can be found (Vogel et al., 2025).

Chemotherapeutic agents can be applied locally by Trans arterial chemoembolization (TACE) to highly vascularized HCC, allowing delivery of higher concentrations of the cytostatic drug to tumor and minimizing systemic side effects. However, classical chemotherapy is used as a palliative approach because HCC has low response rates (Befeler & di Bisceglie, et al.,2002). In HCC patients, antitumor drugs as chemotherapeutic agents such as sorafenib or tivantinib have already been used (Wild et al., 2013). HCC is a relatively radiosensitive tumor. Although radiotherapy has not been fully implemented in the regimen of HCC therapy, some studies showed its useful application against the highly aggressive radiosensitive tumor (Bang & Dawson, et al., 2019).

Importantly, one of the most common manifestation of HCC and side effects of the therapy is cancer related fatigue (CRF), which may be due to disruption of sleep/wake cycle and activity rhythms. Thus, studying the influences of HCC on the circadian system is crucial for understanding the systemic effects of cancers and for designing appropriate treatment approaches.

1.2 The circadian system

In mammals, internal timekeeping, which is important for anticipating rhythmic processes in the environment and thus for optimized rhythmic bodily functions, is ensured by a hierarchically organized circadian system. The central element is the circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus (Figure 1). It generates rhythms with a period of approximately 24 hours and receives a light input via the retinohypothalamic tract which the phase and period of these circadian rhythms are

synchronized with external time, a process known as entrainment (Reppert & Weaver, 2002; von Gall, 2022). The SCN controls subordinate oscillators in the central nervous system (CNS) and periphery, primarily via its connection to the paraventricular nucleus, a control center for the autonomic nervous system and the endocrine system.

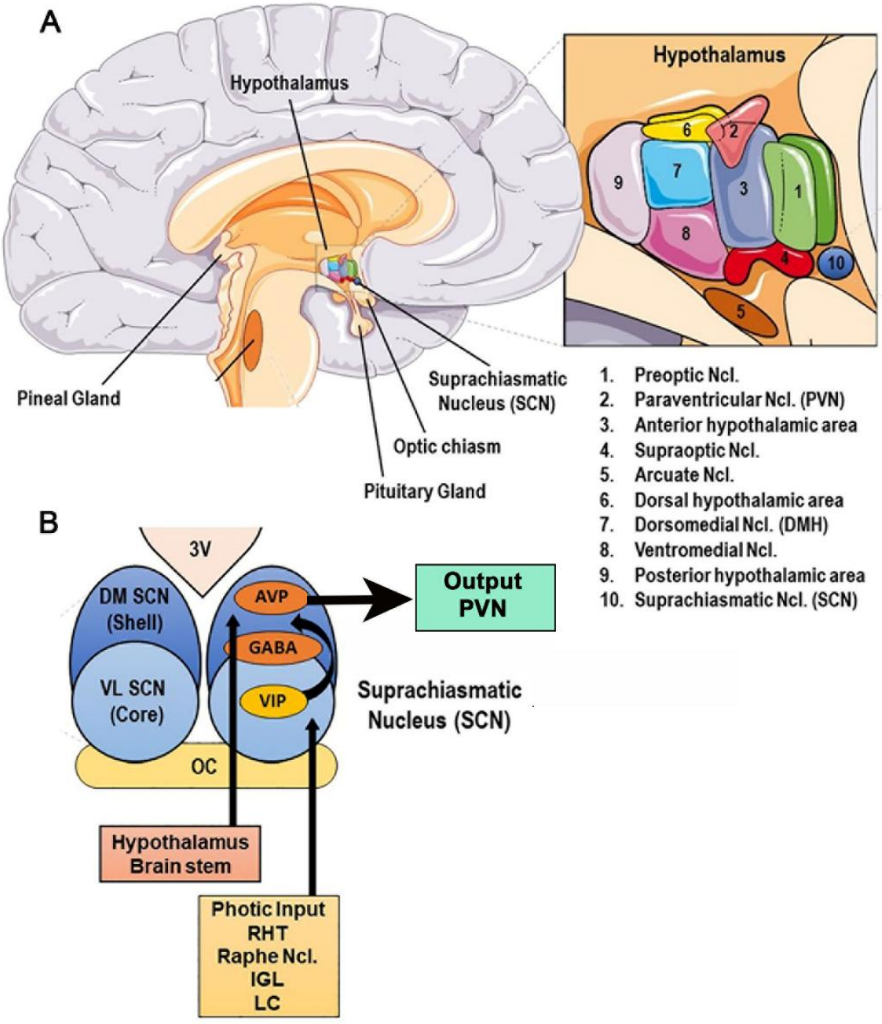

Fig. 1 The suprachiasmatic nucleus (SCN) (A) Location within the hypothalamus of humans. (B) Subregions of the SCN and their input and output connections. The ventrolateral (VL) core region contains neurons characterized by the expression of vasoactive intestinal peptide (VIP) and receives photic input from the retina via the retinohypothalamic tract (RHT) and from other brain regions. The dorsomedial (DM) shell region contains neurons characterized by the expression of arginine vasopressin (AVP) and sends output to other hypothalamic regions particularly to the paraventricular nucleus. All SCN neurons use the neurotransmitter γ-aminobutyric acid (GABA) for coupling. 3V, third ventricle; IGL, thalamic intergeniculate leaflet; oc, optic chiasm, LC locus caeruleus (adapted from Agorastos et al., 2020).

1.2.1 Light input to the circadian pacemaker

The light is received by the retinal photoreceptors. Rods are specialized to detect light at low intensity as they contain rhodopsin and thus, they are responsible for night vision, while cones are responsible for daylight and colour vision due to their content of photopsins. Stimulation of both types of photoreceptors leads to photo signal transduction to bipolar and horizontal cells and finally to the retinal ganglion cells (RGCs) to mediate the image-forming visual functions (Fu & Yau, 2007).

In addition to the classical photoreceptors, the rods and cones, the retina possesses a subset of RGCs which are intrinsically photosensitive (ipRGCs). ipRGCs contain the photopigment melanopsin, which reacts most sensitively to blue light and play an important role in non-image forming visual function, such as the pupillary light reflex and circadian entrainment (Ecker et al., 2010). The ipRGCs are functionally independent of the rods and cones as transgenic mice lacking rods and cones show non-image forming visual functions (Berson et al., 2002; Güler et al., 2007).

The axons of ipRGCs project directly to the SCN core via the retino-hypothalamic tract (RHT) (Wahl et al., 2019). Light information is transmitted to SCN neurons in the core region by the release of pituitary adenylyl cyclase activating polypeptide (PACAP). Glutamate binds to NMDA and AMPA receptors and activates signalling cascades in the SCN neurons. The signal transduction includes phosphorylation of the cyclic AMP response element-binding protein (CREB) (Ding et al., 1998) and of the ERK1 kinase and expression of the clock gene *Per1 (Gau et al., 2002)* . Light-induced expression of *Per1* leads to resetting of the molecular clockwork in the SCN, and thus to circadian entrainment (von Gall, 2022).

Photic information is also indirectly transmitted the SCN from the intergeniculate leaflet (IGL) via Neuropeptide Y (NPY) and Gamma-aminobutyric acid (GABA) release (Moore & Blanchard, 1992). In addition, the SCN receives input from the raphe nuclei via serotonergic signalling (Figure 1(B)). These inputs can modulate the circadian entrainment and pacemaker function (Smith et al., 2015a,Jacobs & Azmitia, 1992).

1.2.2 The circadian pacemaker

The circadian system in mammals is a hierarchically organized system with the main pacemaker being the SCN in the anterior hypothalamus (Moore et al., 2002). The SCN is formed of paired nucleus adjacent to third ventricle and located above the optic chiasma

(Figure 1(A)). Each is formed of 10000 highly compacted tightly connected neurons in addition to glial cells. The SCN can be broadly divided into two main anatomical areas:
1) the ventrolateral core, which contains vasoactive intestinal peptide (VIP)- (10% of the SCN neurons) and gastrin releasing peptide (GRP)- positive neurons in addition to small populations of neurons that release neurotensin (NT) and calretinin (CT), and
2) the dorsomedial shell, which contains arginine vasopressin peptide (AVP)-producing neurons (20% of the SCN neurons) (Abrahamson & Moore et al., 2001). GABA is colocalized in almost all neurons of the SCN (Moore & Speh, 1993).

The core region is the main area that receives input from the retina, where the terminals of retinohypothalamic tract contact the VIP-positive neurons (Pfaff, et al., 2013). VIP is particularly important for the entrainment of circadian rhythms to the light/dark cycle (Jones et al., 2018; Vosko et al., 2015) and for the synchronization of the neural network within SCN (Ono et al., 2021). Thus, Mice lacking the VIP receptor 2 showed desynchronized temporal rhythms with altered behavioural patterns. (Abrahamson & Moore, et al., 2001). The AVP-expressing neurons play an important role (Yamaguchi et al., 2013) for the synchronization between SCN and other hypothalamic areas (Tousson & Meissl, 2004). The other neurotransmitters are involved in generation and fine tuning of rhythms in the SCN (Webb et al., 2009).

The SCN also includes glial cells like astrocytes and microglia. Astrocytes have important functions e.g. the release of growth factors and regulation of extracellular levels of neurotransmitters such as glutamate and thus preventing excitotoxicity through uptake via special transporters (Sofroniew & Vinters, 2010). Astrocytes can control the oscillation in the SCN and thus also the circadian pattern (Brancaccio et al., 2019a). Glial fibrillary acidic protein (GFAP) belongs to the structural proteins of intermediate filaments mainly found in the soma and stem processes of astrocytes. Reactive astrocytes can be detected in pathological and inflammatory processes in the brain. They differ in their morphological and functional properties from non-reactive astrocytes (Eddleston & Mucke, 1993; Pekny & Nilsson, 2005; Wilhelmsson et al., 2006) and show upregulation of GFAP (Eng et al., 2000).

Microglia are specialized immune cells that reside in the CNS and act as the brain's intrinsic macrophage-like defence system. They continuously survey the neural tissue, remove pathogens and cellular debris by phagocytosis, and help regulate brain development, synaptic pruning, and repair after injury. Microglia possess a molecular

clockwork (Guzmán-Ruiz et al., 2022) and modulate the rhythmicity of SCN neurons, e.g. deletion of microglia leads to a reduced amplitude of rhythmic clock gene expression in SCN neurons (Honzlová et al., 2023a). However, circadian rhythms in locomotor activity appear unaffected by the absence of microglial cells (Matsui et al., 2023). In neurodegenerative diseases, microglial dysfunction is correlated with sleep disorders (Son et al., 2024). The activation of microglial cells in mice has also been detected in tumors such as breast cancer (Lawther et al., 2022a). The most commonly used marker for microglial cells is IBA1 (Lier et al., 2021). The activation of both astrocytes and microglial cells leads to the generation of oxidative stress (Chen et al., 2020; Iizumi et al., 2016).

1.2.3 The molecular clockwork

The basis for the generation of circadian rhythms of gene expression, and thus of cell function, is the molecular clockwork (Fig. 2). It is found not only in the cells of the SCN but in almost all body cells. While the molecular clock in the SCN is set by light (see above) and is very stable and self-sustaining, it is triggered in the subordinate oscillators by rhythmic neuronal and endocrine signals (Pfaff, et al., 2013, Yamazaki et al., 2000).

The molecular clock consists of transcriptional/translational feedback loops of clock genes that encode transcription factors and negative transcriptional regulators. The transcription factors Bmal1 and CLOCK form a positive regulatory complex that, by binding to E-box promoter elements, activates the expression of *Pers* and *Crys* as well as clock-controlled genes (CCG). *Pers* and *Crys* form a negative regulatory complex that inhibits the activity of the positive regulatory complex and thus the expression of clock-controlled genes as well as their own expression (Lee et al., 2001). The negative regulatory complex is eliminated via degradation and thus the cycle starts again after approximately 24 hours. In addition, the rhythmic expression of Bmal1 is controlled by a second feedback loop through the transcription repressor Rev-Erba and the enhancer Rora (Preitner et al., 2002).

The rhythmic expression of the CCG is controlled by the molecular clockwork and essential for rhythmic cell function. Multi-organ transcriptome data indicate that 40–50 % of the mammalian genome is under circadian control (Vitaterna et al., 2019)

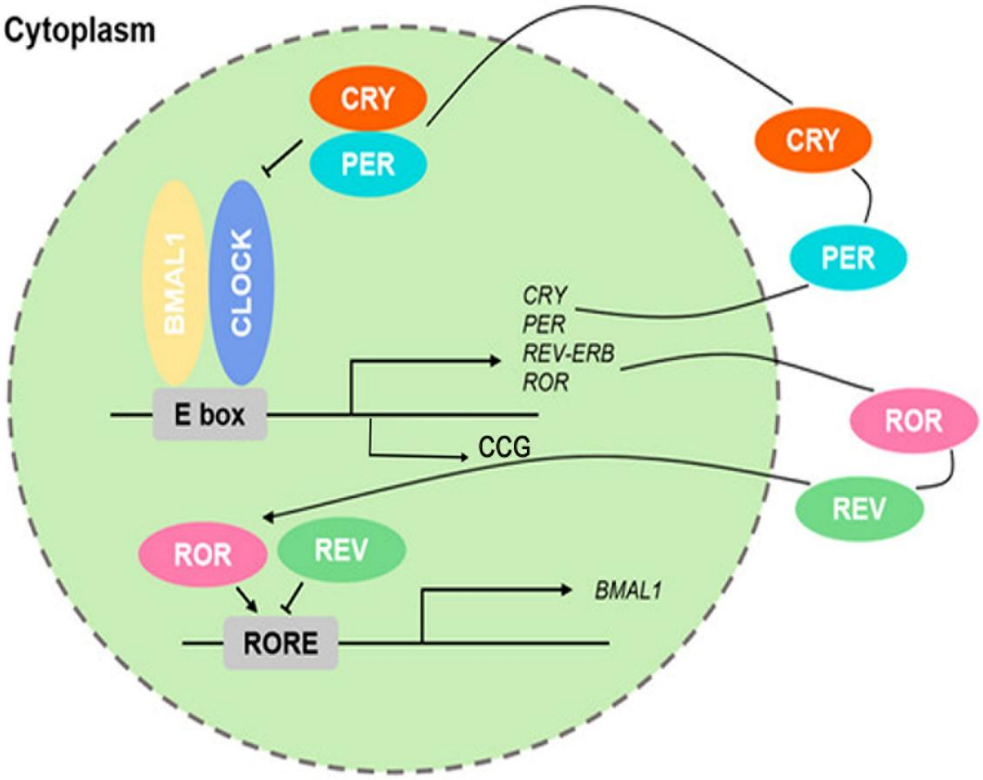

Fig. 2 The core mammalian molecular clockwork generates a circadian rhythm CLOCK and BMAL form a complex that activates the transcription of the Pers and Cry as well as clock controlled genes (CCG). When the Per and Cry proteins are formed, they form a complex which, after translocation into the nucleus, inhibits the CLOCK/Bmal1 complex. After degradation of Pers and Crys, a new cycle can start. ROR and REV Erba control rhythmic Bmal1 expression (adapted from Mao et al., 2025).

1.2.4 SCN Output

The SCN controls rhythmic functions in behavior and physiology by controlling rhythmic neuronal and endocrine signals for subordinate circadian oscillators in the brain and periphery (Morin, 2007; Morin et al., 1994) (Figure 3). The SCN neurons send rhythmic neuronal signals directly to the PVN, which controls the endocrine and autonomic nervous systems (Buijs et al., 2003; Kalsbeek et al., 2006). Rhythmic endocrine signals of the circadian system are primarily melatonin and cortisol (Buijs et al., 2003; Kalsbeek et al., 2006). The rhythmic synthesis of the dark hormone melatonin in the pineal gland, one of the best-studied rhythmic processes in the circadian system, is controlled by the autonomic nervous system (Kalsbeek & Buijs, 2002a; Von Gall et al., 1998). The rhythmic signals from the SCN control the rhythmic activity of the preautonomous

neurons in the PVN. These project to the intermediolateral column in the spinal cord. From there, the preganglionic fibers project to the superior cervical ganglion of the sympathetic trunk. From there, the postganglionic fibers project to the pineal gland (Drijfhout et al., 1996) to promote the rhythmic synthesis of melatonin in the dark phase (Klein & Weller, 1970). Melatonin can modulate the SCN and subordinate oscillators in the brain and the periphery (Pfeffer et al., 2022). By binding to G-protein-coupled MT1 and MT2 receptors and signaling pathways, melatonin can modulate the phase in the rhythmic transcription of clock genes and CCG (Richards & Gumz, 2012) and inhibit neuronal activity of SCN neurons (Hernández-Rabaza et al., 2016a, 2016b). The most common laboratory mouse strain, C57BL/6, is melatonin-deficient but exhibits mostly intact circadian rhythms. However, melatonin plays a major role in seasonal rhythms (Pfeffer et al., 2022).

The rhythmic synthesis of cortisol is controlled by the hypothalamo-pituitary adrenal (HPA) axis. The rhythmic signals from the SCN control the rhythmic activity of the corticotropin-releasing hormone (CRH) producing neurons in the PVN (Kalsbeek & Buijs, 2002b). Cortisol is not only a stress hormone but also an important rhythmically produced hormone that plays a central role in the circadian system for the synchronization of subordinate oscillators in the brain and periphery (O'Byrne et al., 2021). Therefore, changes in cortisol levels during inflammatory processes and/or tumors can also alter circadian rhythms. This has already been demonstrated for changes in the sleep/wake cycle in mice with HCC (Hassan, Ali, Yassine, Sohn, Pfeffer, Jänicke, H.-W. Korf, et al., 2021; Huang et al., 2020). Similarly, patients with colorectal cancer have a significantly reduced activity pattern and higher serum levels of cytokines such as TNF-α, as well as a disturbed cortisol rhythm. This suggests that inflammatory processes in humans can also alter rhythmic processes by changing cortisol levels (Rich et al., 2005).

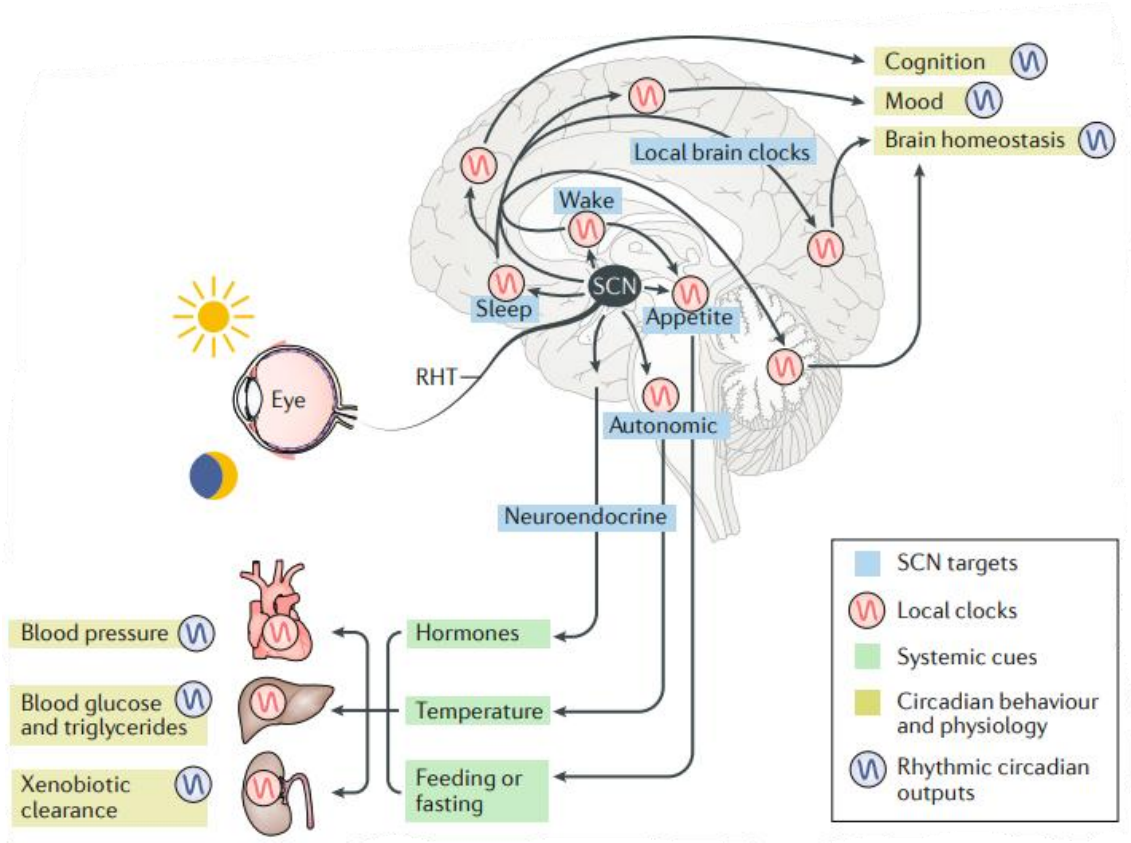

Fig. 3 Rhythmic signals control rhythmic body functions. Information about the light-dark cycle of the environment is transmitted to the eye via the retinohypothalamic tract (RHT) directly to the SCN. The SCN acts via rhythmic output to other hypothalamic brain regions to regulate sleep and wakefulness, body temperature, appetite, hormone secretion, and the activity of the autonomous nervous system. Rhythms of cognitive function are either directly controlled by the light-dark cycle or indirectly controlled by involving the SCN. Rhythms in systemic cues such as hormones, temperature and food intake modulate circadian oscillators in various organs producing rhythms in physiology such as metabolic, immune and cardiovascular function (adapted from Hastings et al., 2018).

1.3 The Liver-Brain axis

Chronic liver diseases can impact the central nervous system. For instance, patients with liver cirrhosis may suffer from depression and cognitive deficits, associated with changes in the homeostasis of neurotransmitters such as serotonin (Smith et al., 2024). Hyperammonaemia and neuroinflammation can trigger hepatic encephalopathy, which is associated with increased oxidative stress and manifests as neuronal dysfunction, including impairment of brain oscillators (Görg et al., 2010; Häussinger et al., n.d.) Similarly, bile duct ligation (BDL) is associated with anxiety disorders, depression and memory impairment probably due to increased expression of pro-inflammatory cytokines and decreased serotonin (Jiang et al., 2018).

The autonomic nervous system is also involved in the communication between the liver and the brain (Matsubara et al., 2022) (D'Mello & Swain et al., 2011). Inflammatory cytokines, such as IL-1, can activate IL-1 receptors on terminals of the vagus nerve that innervate the liver and can retrogradely transmit signals to the brain, leading to altered neuronal activity in key brain structures such as the PVN. In line with this, intraperitoneal administration of cytokines in mice with vagotomy did not lead to behavioural changes (Bluthé et al., 1996).

Furthermore, TNF-a and IL-1 receptors are expressed in cerebral endothelial cells and their activation leads to upregulation of signalling molecules such as nitrit oxides (NO), which can affect neuronal function. Furthermore, in some brain regions, the so-called circumventricular organs, an intact blood-brain barrier is lacking, allowing cytokines to act directly and inflammatory cells to more easily penetrate the brain (Hunt et al., 2022; Matsubara et al., 2022; Swain & Jones, 2019).

Importantly, the microglia represent the immune cells of the brain, which are activated by inflammation-induced cytokine. Liver diseases associated with hyperammonaemia, has been proven to increase neuro-immune activity and cause changes in the hippocampus and its function, such as spatial memory (Hernández-Rabaza et al., 2016b). Moreover, it has been shown that inflammatory liver disease can lead to the infiltration of monocytes into the brain in mice (D'Mello et al., 2009), which may be responsible for the associated symptoms, including fatigue, cognitive dysfunction and exhaustion (D'Mello & Swain, 2014). (D'Mello & Swain, 2011)(York et al., 2012). Besides, liver growth factor, which is increasingly released in liver diseases, promotes microglial activation and reactive

astrocytogenesis (Gonzalo-Gobernado et al., 2009)(Jayakumar & Norenberg, 2013; Pekny & Pekna, 2014; Tilg & Diehl, 2000).

In summary, chronic liver diseases could impact the central nervous system via neural and humoral pathways. Understanding how the liver communicates with the brain in the chronic liver diseases will help define novel treatment targets that can decrease the burden of chronic liver diseases.

1.4 Cancer and circadian rhythms

Chronic disruption of the circadian rhythms is considered a risk factor for the development of cancer. On the other hand, cancer could lead to fatigue and sleep disorders, which subsequently impact the circadian system (Kisamore et al., 2024). This reciprocal interaction has recently acquired a major scientific attention.

The circadian system not only regulates the sleep-wake rhythm but also influence multiple cellular processes such as cell cycle and DNA repair, which suggests that disturbances in circadian rhythms could promote the development and spread of cancer (López-Otín & Diamandis, 1998). Besides, the circadian system influences the hormonal production and immunity and disturbed rhythms, for example jet lag, sleep deprivation, or shift work can result in enhanced release of cytokines leading to chronic inflammation and, thus, promote the development of cancer (Padilla et al., 2024). Interestingly, melatonin, known as the hormone of darkness/sleep, leads to a resynchronization of clock gene expression to tumor-inhibiting properties (Otálora et al., 2008), and thus, its dysfunction may increase the risk of cancer development.

On the other hand, cancer could lead to fatigue and sleep disorders, which subsequently impact the circadian system via neural and humoral pathways (see 1.3.)

Recently, chronotherapy, which means timed administration of medication, has gained an increasing scientific significance to achieve the highest effectiveness and the lowest side effects. This concept is based on circadian regulation of metabolism of pharmaceuticals by regulation of enzymes responsible for the degradation of drugs (Lin et al., 2019) as well as various cell functions e.g. proliferation and DNA repair (Hassan, Ali, Yassine, Sohn, Pfeffer, Jänicke, H.-W. Korf, et al., 2021).

For example, antihypertensive drugs such as Angiotensin-Converting Enzyme (ACE) inhibitors had a greater effect when taken in the evening (Hermida et al., 2011), while administration of glucocorticoids for treatment of rheumatoid arthritis in the evening reduced the symptoms of morning stiffness (Buttgereit et al., 2010).

Chronotherapy has also been considered an important approach in cancer treatment. Anticancer drugs may have the greatest antiproliferative effect on the tumor cells when applied at specific time-of-day (Levi et al., 2010). For instance, Application of 5- Flour-Uracil (5-FU) as a treatment for prostate carcinoma at 4:00 a.m. shows 10 times fewer toxic effects compared with a flat long-term infusion least toxicity. 6-Mercaptopurine and methotrexate were found to be significantly better as evening medications (Hsu et al., 2016). Moreover, administration of anticancer drugs in the morning for breast cancer patients resulted in a worse outcome (Johnson et al., 2019). Furthermore, there is a time-of-dependant effect of radiotherapy for the treatment of HCC in mice on liver cells and on the hippocampus (Hassan, Ali, Sohn, et al., 2021).

1.5 Aim of the Study

Cancer and liver damage can impair various brain functions. However, there is currently little evidence on how they affect the circadian system. The aim of this study was therefore to investigate the influence of HCC on the central circadian pacemaker in the SCN. In SCN of HCC bearing mice, we investigated the key neuropeptides (VIP and AVP), an essential component of the molecular clockwork (Bmal1), markers for activity of neurons (c-Fos), astrocytes (GFAP), microglia (IBA1), as well as oxidative stress (8-OHdG). Data obtained in this study can contribute to a better understanding of chronodisruption in cancer/liver diseases and support the development of therapeutic strategies to obtain better treatment outcomes and improve the quality of life of the patients.

2 Related Publication:

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Hepatocellular Carcinoma in Mice Affects Neuronal Activity and Glia Cells in the Suprachiasmatic Nucleus

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Abstract: Background: Chronic liver diseases such as hepatic tumors can affect the brain through the liver–brain axis, leading to neurotransmitter dysregulation and behavioral changes. Cancer patients suffer from fatigue, which can be associated with sleep disturbances. Sleep is regulated via two interlocked mechanisms: homeostatic regulation and the circadian system. In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is the key component of the circadian system. It generates circadian rhythms in physiology and behavior and controls their entrainment to the surrounding light/dark cycle. Neuron–glia interactions are crucial for the functional integrity of the SCN. Under pathological conditions, oxidative stress can compromise these interactions and thus circadian timekeeping and entrainment. To date, little is known about the impact of peripheral pathologies such as hepatocellular carcinoma (HCC) on SCN. **Materials and Methods**: In this study, HCC was induced in adult male mice. The key neuropeptides (vasoactive intestinal peptide: VIP, arginine vasopressin: AVP), an essential component of the molecular clockwork (Bmal1), markers for activity of neurons (c-Fos), astrocytes (GFAP), microglia (IBA1), as well as oxidative stress (8-OHdG) in the SCN were analyzed by immunohistochemistry at four different time points in HCC-bearing compared to control mice. **Results**: The immunoreactions for VIP, Bmal1, GFAP, IBA1, and 8-OHdG were increased in HCC mice compared to control mice, especially during the activity phase. In contrast, c-Fos was decreased in HCC mice, especially during the late inactive phase. **Conclusions**: Our data suggest that HCC affects the circadian system at the level of SCN. This involves an alteration of neuropeptides, neuronal activity, Bmal1, activation of glia cells, and oxidative stress in the SCN.

Keywords: HCC; SCN; circadian system; VIP; AVP; glia; oxidative stress

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer worldwide and causes approximately one million deaths annually [1]. Recently, there has been an increasing number of HCC patients globally [2], often diagnosed at advanced stages after serious consequences have occurred due to the absence of specific symptoms and lack of diagnostic markers [3]. Non-specific symptoms of HCC include fatigue and sleep disturbance [4,5]. Generally, cancer-related fatigue (CRF) is caused by multiple central and peripheral factors, including neurotransmitter imbalance, systemic inflammation, and circadian disruption [6] that negatively impacts the patient's quality of life [5].

The hierarchically organized mammalian circadian system controls rhythms in behavior and physiology, such as activity, sleep, body temperature, hormone release, and food intake [7,8]. It contains a master circadian oscillator that generates an endogenous rhythm of approximately 24 h (circadian), input paths for the entrainment of the circadian rhythms with rhythmic external environmental cues, and output paths for the entrainment of subordinate oscillators. The master circadian oscillator resides in the hypothalamic suprachiasmatic nucleus (SCN) [9]. Rhythmic SCN activity is entrained to the light/dark cycle through the retino-hypothalamic tract (RHT). The integrated time information is transmitted via neuronal and endocrine connections to subordinate oscillators in the brain and the periphery. The SCN can be divided into two functionally different subregions: the ventrolateral core and the dorsomedial shell. Both regions use GABA as a transmitter but in combination with different neuropeptides, the vasoactive intestinal peptide (VIP) in the core region and arginine vasopressin (AVP) in the shell region [10]. The core is innervated by the RHT and innervates the shell, which in turn innervates other key regions of the hypothalamus [11]. At the cellular level, a molecular clockwork composed of transcriptional/translational feedback loops of clock genes controls rhythmic cell function. The transcription factor Bmal1 is an essential component of molecular clockwork and is involved in the generation and entrainment of circadian rhythms in SCN [12,13]. At the SCN cellular network level, the coupling of both neurons and astrocytes [14,15], as well as VIP and AVP [10,16–19], contribute to both the stability and the entrainment of circadian rhythms. Light information is encoded by the release of glutamate and neuropeptides from RHT connecting to VIP neurons in the core. The stimulation of the respective receptors activates the MAPK/CRE-binding pathways and, subsequently, the neuronal activity marker c-Fos and clock gene Per1 to reset the molecular clockwork [20–22].

So far, little is known about the pathomechanisms of structural and functional changes in the brain caused by chronic liver diseases. The liver–brain axis might involve visceral afferent connections, including sympathetic and parasympathetic nervous systems [23] and blood-borne signals such as immune cells, cytokines, and metabolites that reach the brain, particularly through a disease-related deficient blood–brain barrier [1,23,24]. In hepatic encephalopathy, ammonia and inflammation are the major triggers for oxidative stress, astrocytic/neuronal dysfunction, and subsequent disturbances of brain oscillatory networks [25–27]. HCC involves pathologies in liver structure and function associated with chronic inflammation, resulting in hepatocellular DNA damage, and can be recapitulated by the administration of genotoxic agents in animals [28]. HCC is associated with sleep–wake disturbance and poor sleep quality in patients [29]. Higher diurnal cortisol level suggests a disturbed circadian rhythm in HCC patients, which may underlie their poor sleep quality [30].

Clock genes are pivotal players that regulate cell proliferation and apoptosis in HCC [31]. Furthermore, they are considered potential diagnostic markers and therapeutic targets in HCC [32]. HCC affects transcription and posttranscriptional modification of clock genes [32] and, consequently, rhythmic functions also in other tissues [33–35], including the hippocampus [36].

In the hippocampus, HCC-dependent changes in clock genes are associated with upregulation of genes encoding for proinflammatory cytokines and reduced proliferation of neuronal progenitors [36], suggesting that liver cancer affects the molecular clockwork, the innate immune system, and adult neurogenesis in the brain. Moreover, we showed earlier that HCC is associated with changes in corticosterone rhythms, locomotor activity (reminiscent of fatigue), and pERK immunoreaction in SCN [37], suggesting that the circadian system is compromised by liver cancer. The aim of this study was to better understand the effect of HCC on the circadian system. We investigated the neuropeptides, SCN neuronal and glial activity, molecular clockwork, and oxidative stress in the context of HCC.

2. Materials and Methods

2.1. Animal Experiments and Tissue Preparation

The animal experiments were approved by the local authority, the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Reference number: AZ 81-02.04.2018-A146) and were conducted according to Directive 2010/63/EU of European Parliament and the Council on the Protection of Animals Used for Scientific Purposes. The experimental design is described in detail in [35–37]. Briefly, 14-day-old male Per2::Luc mice with a C57BL6/J background were randomly divided into an experimental group and a control group. In the experimental group, HCC was chemically induced by a single intraperitoneal injection of the genotoxic agent diethylnitrosamine (10 mg/kg, Cat # N0756-10ML, Sigma Aldrich, Burlington, MA, USA). Phenobarbital (PB) was chronically added to the drinking water (0.05%, Luminal, Desitin, Hamburg, Germany) to enhance HCC development. The control group was only given PB in the drinking water (PB control). At the age of one month, mice were separated from their mothers, and litters were kept in groups of 3–4 mice per cage under 12 h light/12 h dark. During the light phase, the light intensity was 300 lx. Zeitgeber time (ZT) 00 is defined as the time when light was turned on (06:00 am), while ZT12 is defined as the time when light was turned off (06:00 pm). At the age of 7–8 months, mice were sacrificed at four different time points at six-hour intervals, starting at ZT02, thus two hours after lights were on (08:00 am), ZT08: eight hours after lights were on, ZT14: two hours after lights were off and ZT20: eight hours after the lights were off. This provides us with tissue collected at 2 different time points during the early and late light phase (ZT02, ZT08, respectively), and 2 different time points during the early and late dark phase (ZT14, ZT20) to allow the comparison between these time points and phases.

Mice were injected intraperitoneally with a mixture of ketamine (100 mg/kg, Actavis, Ireland) and xylazine (10 mg/kg, Bayer, Leverkusen, Germany), then were intracardially perfused with 0.9% NaCl, followed by 4% formalin, using a peristaltic pump (World Precision Instruments, Sarasota, FL, USA). For mice sacrificed during the dark phase, perfusion and tissue collection were performed exclusively under dim red light.

The livers were inspected for tumors (Figure S1). Of the experimental group, only the mice of comparable tumor size were included (n = 12), while animals with no visible or too small lesions were excluded (n = 4). Of the PB control group, all mice could be included because none had a tumor (n = 12). The brains were extracted, post-fixed in 4% formalin for additional 24 h, cryoprotected in 30% sucrose, and snap frozen. The brains were cryo-sectioned into series of free-floating 30 µm thick coronal sections. Series of sections, including the SCN from these animals, were used for immunohistochemical staining in our previous work [37], while the other series were used here to obtain our novel data published here.

2.2. Immunohistochemistry

All sections used for each marker were processed together at one staining set to avoid the variability in staining conditions.

2.2.1. Chromogenic Reaction

Step 1: Serial brain sections were washed with phosphate-buffered saline (PBS). Step 2: Sections were incubated with 0.6% H_2O_2 for 30 min, followed by washing with PBS containing 0.2% Triton (PBST). Step 3: Sections were incubated in PBST containing 5% normal goat serum (PBSTN) for one hour, followed by incubation with primary antibodies (Table 1) at 4 °C overnight. Step 4: The sections were incubated with the corresponding IgG-biotin-conjugated secondary antibody (Table 2) in PBSTN for one hour at room temperature. This was followed by washing with PBST and incubation with avidin/biotin-based peroxidase system (ABC kit, Vector Laboratories, Newark, CA, USA) for one hour. The sections were rinsed and, finally, incubated with 0.05% 3,3′-diaminobenzidine (DAB) for 5 min. Step 6:

Sections were rinsed with PBS, mounted on slides, and cover slipped using DePeX (Serva Electrophoresis, Heidelberg, Germany).

Table 1. Primary antibodies.

Antibody	Manufacturer	Concentration
Anti-VIP (rabbit, monoclonal, cat #4245)	BMA Biomedicals (Augst, Switzerland)	1:4000
Anti-AVP (rabbit, polyclonal, cat # AHP372)	BIO-RAD (Berkeley, CA, USA)	1:1000
Anti-Bmal1 (rabbit, monoclonal, cat #14268-1-AP)	Proteintech (Rosemont, IL, USA)	1:500
Anti-orexin (rabbit, monoclonal, cat # 16743)	Cells Signaling Technology (Danvers, MA, USA)	1:2000
Anti-IBA1 (rabbit, polyclonal, cat # 019-19741)	WAKO (Osaka, Japan)	1:2000
Anti-GFAP (mouse, monoclonal, cat # 556330)	BD Biosciences (Eysins, Switzerland)	1:500
Anti-8-OHDG (mouse, monoclonal, cat #AM03160PU-N)	ORIGENE (Herford, Germany)	1:1000
Anti-c-Fos (rabbit, monoclonal, cat #4384)	Cells Signaling Technology (Danvers, MA, USA)	1:1000

Table 2. Secondary antibodies.

Antibody	Manufacturer	Concentration
Anti-rabbit IgG Biotin (goat, cat # BA-1000)	Vector Laboratories (Burlingame, CA, USA)	1:500
Anti-rabbit IgG Alexa Fluor 488 (goat; cat # A-11036)	Molecular Probes (Eugene, OR, USA)	1:500
Anti-mouse IgG Alexa Fluor 568 (goat; cat # A-11031)	Molecular Probes (Eugene, OR, USA)	1:500

2.2.2. Immunofluorescence

The steps 1, 3, and 6 were performed as described in Section 2.2.1. Step 2 was omitted. Step 4 was performed as follows: sections were incubated with the corresponding IgG-AlexaFluor-conjugated secondary antibody (Table 2) diluted in PBST containing 5% normal goat serum for one hour at room temperature.

2.2.3. Antibody Specificity

The specificity of primary antibodies has been confirmed previously [38–44] and was also evident by cell morphology, subcellular localization, and region-specific distribution. The specificity of secondary antibody was confirmed by treating the sections as in Sections 2.2.1 and 2.2.2 described, but without adding primary antibody. To test the specificity of IgG-biotin-conjugated secondary antibody, sections were incubated with 0.6% H_2O_2 for 30 min and then in PBST containing 5% PBSTN at 4 °C overnight. The sections were incubated with IgG-biotin-conjugated secondary antibody in PBSTN for one hour at room temperature, followed by washing and incubation with avidin/biotin-based peroxidase system for one hour. The sections were rinsed and, finally, incubated with DAB for 5 min, then mounted on slides and cover slipped. To test the AlexaFluor-conjugated secondary antibody, the previous steps were repeated without incubation in 0.6% H_2O_2. The specificity was confirmed as a positive staining signal was not detected.

2.3. Image Acquisition and Analysis

Coronal sections containing the hypothalamus were imaged using ×20 objective of a Keyence BZ-X800 microscope (Keyence, Osaka, Japan). During image acquisition and processing, all settings, including illumination intensity and step size, were kept constant for each staining set. The analysis was performed by an investigator blind to the experimental conditions. Image analysis was performed using ImageJ (Version 1.53a) software [45]. This automated analysis helps obtain standardized and accurate quantification of staining. The SCN was demarcated in the coronal brain sections due to the presence of densely packed nuclei dorsal to the optic chiasm on both sides of the lateral ventricle in the hypothalamic area from Bregma −0.34 to −0.7 [46]. Boundaries were traced using ImageJ's freehand tool (Figure S2A). The immunoreactions were analyzed in the middle part of the rostrocaudal extent in the right and left SCN from 1 to 2 sections per mouse and then averaged to obtain one value per animal. The intensity of background signal in the adjacent neuropil was subtracted from the respective intensity of immunostaining for VIP, AVP, Bmal1, glial fibrillary acidic protein (GFAP), IBA1, and 8-hydroxydeoxyguanosine (8-OHdG) in the SCN, and the resulting intensity was given in arbitrary units (A.U.). The number of c-Fos-immunoreactive cells in the delineated SCN area was counted and given in cells/mm^2. The lateral hypothalamus was demarcated in the coronal brain sections due to the presence of orexin-immunoreactive neurons in the area from Bregma −1.22 to −2.18 [46]. Boundaries were traced using ImageJ's freehand tool (Figure S2B). The number of orexin-immunoreactive cells in the delineated area of the lateral hypothalamus was counted and given in cells/mm^2.

2.4. Statistical Analysis

GraphPad Prism 8 software was used for statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was used to examine the effect of time and HCC and their interaction, followed by Sidak's multiple comparisons test. The results were regarded as significant when p value was <0.05.

3. Results

3.1. Effect of HCC on Neuropeptides in SCN and Lateral Hypothalamus

VIP is expressed by neurons in the SCN core region and is crucial for synchronization of the neuronal network within the SCN [10]. VIP-immunoreactive neurons were found in the SCN of both groups (Figure 1A–H). Their cell bodies were found primarily in the core region, while their axons extended into the shell region (Figure S3A–C). Two-way ANOVA showed an effect of HCC ($F(3,16) = 12.3$, $p = 0.003$). Post hoc multiple comparisons showed a significantly higher intensity of VIP-immunoreaction (IR) in HCC mice than in PB controls in the late dark/activity phase (ZT20, $p = 0.04$) (Figure 1D,H,I).

AVP is also a synchronizer for SCN neurons and plays an important role in determining the period length and the anticipatory behavior [10]. AVP-immunoreactive neurons and axons were found in SCN of both groups, mainly in the shell regions (Figures 2A–H and S3D,E). Two-way ANOVA showed an effect of HCC ($F(3,16) = 18.7$, $p = 0.0005$) ($F(3,16) = 0.5$, $p = 0.66$). Post hoc multiple comparisons showed a significantly higher intensity of AVP-IR in HCC mice than in PB controls in the late dark/activity phase (ZT20, $p = 0.03$) (Figure 2D,H,I).

Orexin-immunoreactive neurons in the lateral hypothalamus play an essential role in the control of the sleep–wake cycle and the locomotor activity downstream of SCN. Two-way ANOVA showed no effect of HCC ($F(3,16) = 0.7$, $p = 0.59$) on the number of orexin-immunoreactive neurons in the lateral hypothalamus (Figure 3A–I).

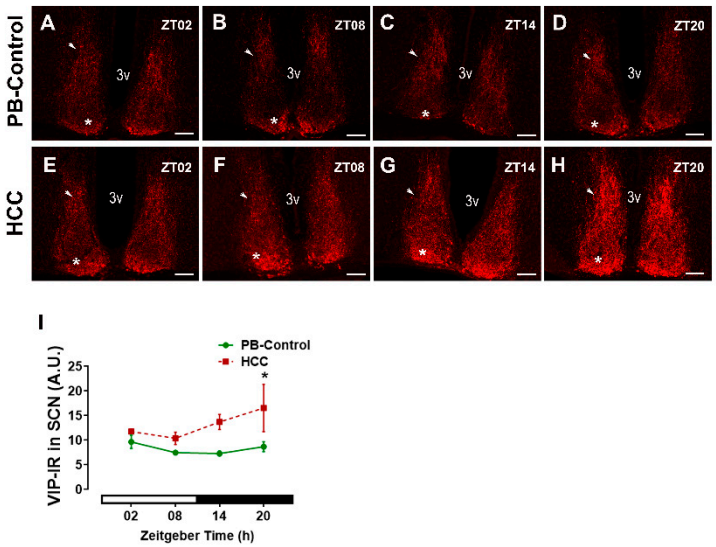

Figure 1. Vasoactive intestinal peptide (VIP) immunoreaction (IR) in the SCN. Representative fluorescent microphotographs showing the immunoreaction (IR) of VIP (red) in the suprachiasmatic nucleus (SCN) of (**A**–**D**) PB control mice and (**E**–**H**) HCC mice. The white asterisk indicates the ventral core region, while white arrowhead indicates the dorsal shell region of SCN. 3v: third ventricle. Scale bar = 100 µm. Mice were sacrificed at different time points at 6 h intervals starting at Zeitgeber time (ZT) 02 = 2 h after the light on. (**I**) Quantification of VIP immunoreaction (IR) in arbitrary unit (A.U.) in the SCN. White and black bar indicates light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point. *: $p < 0.05$ between PB control and HCC.

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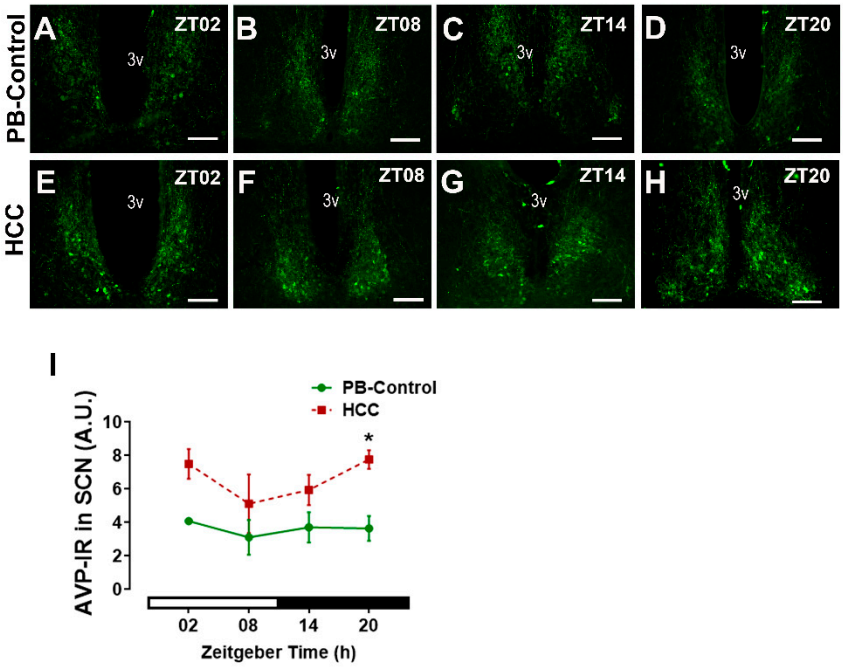
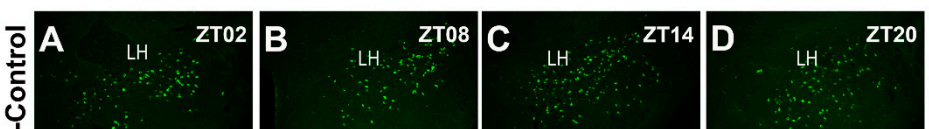


Figure 2. Arginine vasopressin (AVP) immunoreaction (IR) in the SCN. Representative fluorescent microphotographs showing the immunoreaction (IR) of AVP (green) in the SCN of (**A**–**D**) PB control mice and (**E**–**H**) HCC mice. 3v: third ventricle. Scale bar = 100 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light was on. (**I**) Quantification of AVP immunoreaction (IR) in arbitrary unit (A.U.) in the SCN. White and black bar indicates light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point. *: $p < 0.05$ between PB control and HCC.



trol mice and (**E–H**) HCC mice. 3v: third ventricle. Scale bar = 100 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light was on. (**I**) Quantification of AVP-immunoreaction (IR) in arbitrary unit (A.U.) in the SCN. White and black bar indicates light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point. *: $p < 0.05$ between PB control and HCC.

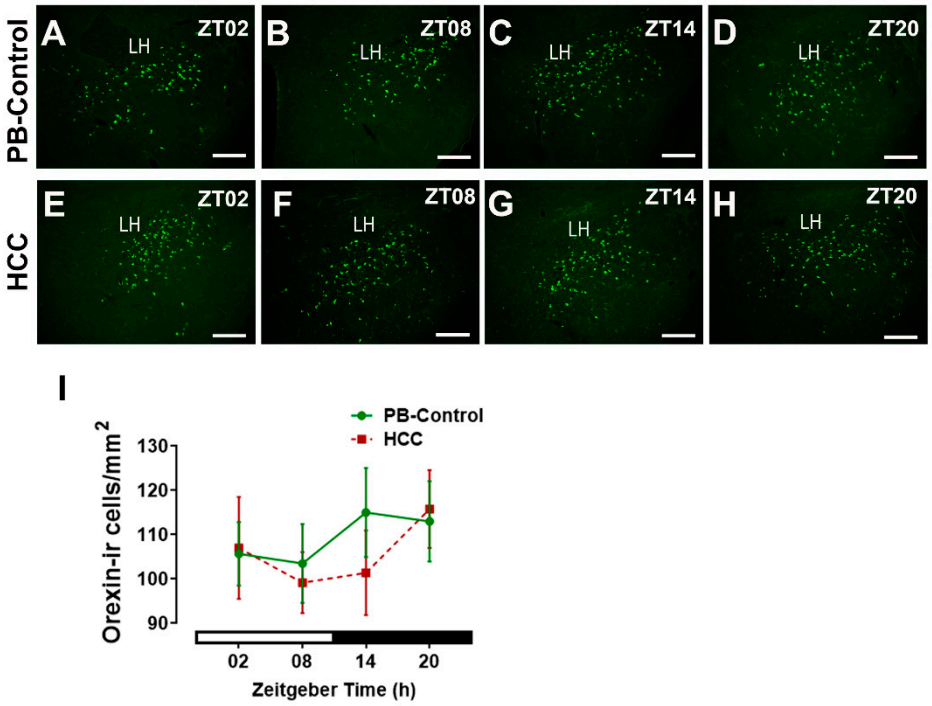


Figure 3. Orexin-immunoreactive (ir) cells in the lateral hypothalamus. Representative fluorescent microphotographs showing orexin-ir cells (green) in the lateral hypothalamus (LH) of (**A–D**) PB control mice and (**E–H**) HCC-bearing mice. Scale bar = 200 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light was on. (**I**) Quantification of number of orexin-immunoreactive cells per mm^2 in the lateral hypothalamus. White and black bar indicates light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point.

3.2. Effect of HCC on Bmal1-Immunoreactive Cells in SCN

Immunoreactive cells for the essential circadian clock protein Bmal1 (Figure 4A–H) showed no differences in the distribution between the two SCN subregions. Two-way ANOVA showed an effect of HCC ($F(3,16) = 13.7$, $p = 0.002$) ($F(3,16) = 2.0$, $p = 0.16$). Post hoc multiple comparisons showed a significantly higher intensity of Bmal1-IR in HCC mice than in PB controls in the late dark/activity phase (ZT20, $p = 0.006$) (Figure 4D,H,I).

3.3. Effect of HCC on the Neuronal Activity Marker c-Fos in SCN

The immunoreaction of c-Fos, the neuronal activity marker, was found in SCN cells of both groups (Figure 5A–H). Two-way ANOVA showed an interaction between time of day and HCC ($F(3,16) = 5.2$, $p = 0.02$). Post hoc multiple comparisons showed a significantly lower number of cFos-immunoreactive cells in HCC mice than in PB controls in the late light/inactive phase (ZT08, $p = 0.04$) (Figure 5B,F,I).

3.4. Effect of HCC on Astrocytic Marker GFAP in SCN

To investigate if HCC affects astrocytes in the SCN, we used the marker of astrocytic processes GFAP, whose increase within brain tissue indicates reactive astrocytes [47] (Figure 6A–H). Two-way ANOVA showed an effect of HCC ($F(3,16) = 19.6$, $p = 0.0004$). Post hoc multiple comparisons showed a significantly higher intensity of GFAP-IR in HCC mice than in PB controls in the late dark/active phase (ZT20, $p = 0.003$) (Figure 6D,H,I).

sons test. Total of 12 mice per group, n = 3 mice at each time point.

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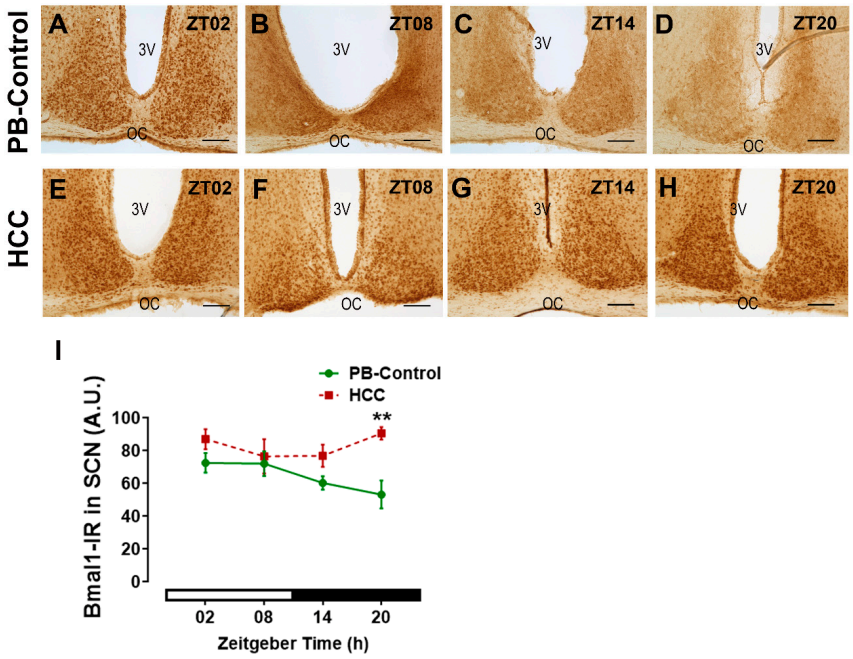

Figure 4. Bmal1-immunoreaction (ir) in the SCN. Representative bright-field photomicrographs showing the Bmal1-immunoreactive cells (brown staining) in the SCN of (**A**–**D**) PB control mice and (**E**–**H**) in HCC-bearing mice. 3v: third ventricle. OC: optic chiasma. Scale bar = 100 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light on. (**I**) Quantification of Bmal1-IR in arbitrary units (A.U.) in the SCN at individual time points at 6 h intervals. White and black bar indicates light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point. **: $p < 0.01$ between PB control and HCC.

3.3. Effect of HCC on the Neuronal Activity Marker c-Fos in SCN

The immunoreaction of c-Fos, the neuronal activity marker, was found in SCN cells [illegible] ween time of [illegible] ed a signifi- [illegible] controls in

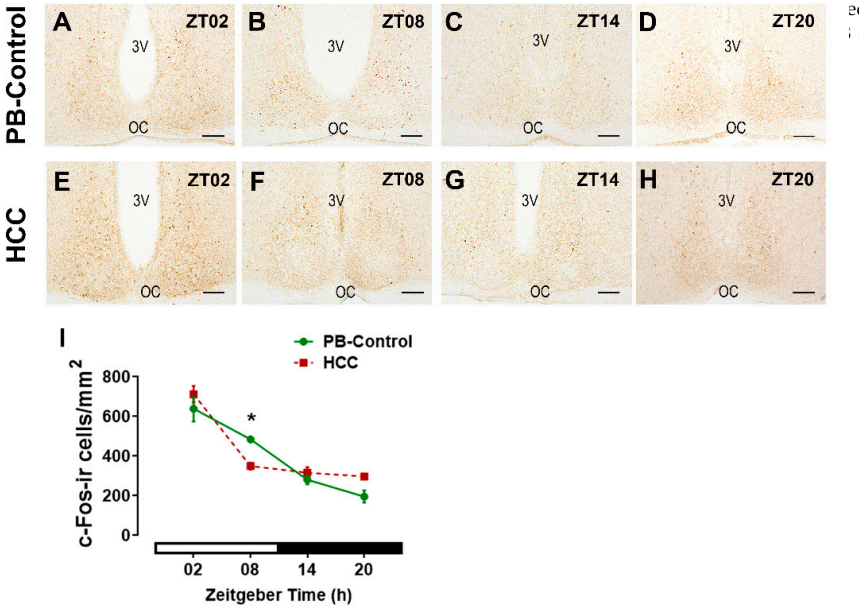

Figure 5. c-Fos-immunoreactive (ir) cells in the SCN. Representative bright-field photomicrograph showing the positively stained c-Fos cells (brown staining) in the SCN of (**A**–**D**) PB control mice and (**E**–**H**) in HCC-bearing mice. 3v: third ventricle. OC: optic chiasma. Scale bar = 100 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light was on. (**I**) Quantification of c-Fos-ir cells/mm^2 in the SCN. White and black bar indicates for light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point. *: $p < 0.05$ between PB control and HCC.

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3.4. Effect of HCC on Astrocytic Marker GFAP in SCN

To investigate if HCC affects astrocytes in the SCN, we used the marker of astrocytic processes GFAP, whose increase within brain tissue indicates reactive astrocytes [47] (Figure 6A–H). Two-way ANOVA showed an effect of HCC ($F(3,16) = 19.6$, $p = 0.0004$). Post hoc multiple comparisons showed a significantly higher intensity of GFAP-IR in

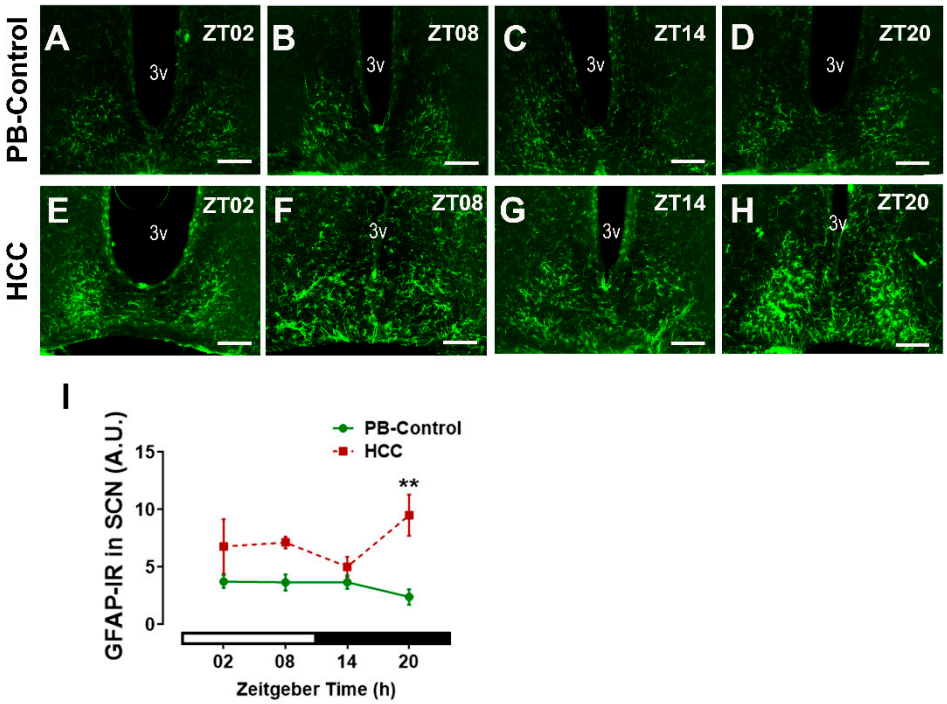

Figure 6. Astrocytic marker GFAP-immunoreaction (IR) in SCN. Representative fluorescent microphotographs showing GFAP immunoreaction (IR) (green) in the SCN of (**A**–**D**) PB control mice and (**E**–**H**) HCC mice. 3v: third ventricle. Scale bar = 150 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light was on. (**I**) Quantification of GFAP-immunoreaction (IR) in arbitrary unit (A.U.) in the SCN. White and black bar indicates light/dark phase, respectively. Two way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group n = 3 mice at each time point. **: $p < 0.01$ between PB control and HCC.

3.5. Effect of HCC on Microglial Marker IBA-1 in SCN

We analyzed whether HCC induced microglia activation in SCN as an inflammatory response by use of IBA1, the most commonly used marker for microglia [48]. IBA1-immunoreactive microglia were detected in the SCN of PB control mice (Figure 7A–D) and the HCC-bearing mice (Figure 7E–H). Two-way ANOVA showed an effect of HCC ($F(3,16) = 16.2$, $p = 0.001$). Post hoc multiple comparisons showed a significantly higher intensity of IBA1-IR in HCC mice than in PB controls in the late dark/active phase (ZT20, $p = 0.002$) (Figure 7D,H,I).

3.6. Effect of HCC on Oxidative Stress Marker 8-OHdG in SCN

To analyze whether HCC induced oxidative stress in SCN, we investigated the DNA oxidative stress marker 8-hydroxydeoxyguanosine (8-OHdG) (Figure 8A–H). Two-way ANOVA showed an effect of HCC ($F(3,16) = 21.7$, $p = 0.0003$). Post hoc multiple comparisons showed a significantly higher intensity of 8-OHdG-IR in HCC mice than in PB controls in the early light/inactive phase (ZT02, $p = 0.03$) (Figure 8A,E,I) and early dark/active phase (ZT14, $p = 0.04$) (Figure 8C,G,I).

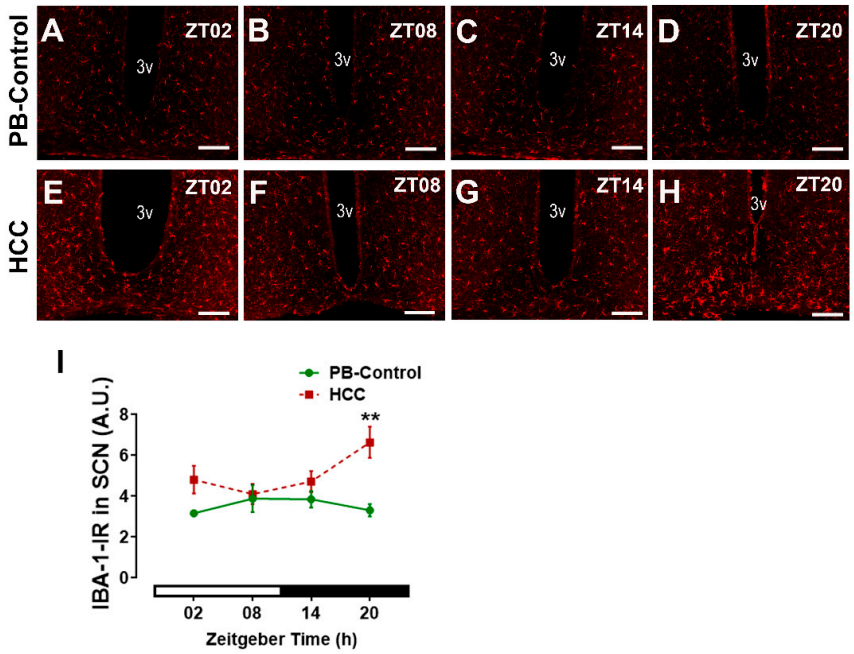

Figure 7. Microglial marker IBA-1-immunoreaction (IR) in SCN. Representative fluorescent photomicrographs showing IBA-1-immunoreaction (IR) (red) in the SCN of (**A**–**D**) PB control mice and of (**E**–**H**) HCC-bearing mice. 3v: third ventricle. Scale bar = 150 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light was on. (**I**) Quantification of IBA-1-immunoreaction (IR) in arbitrary unit (A.U.) in the SCN. White and black bar indicates for light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point. **: $p < 0.01$ between control and HCC.

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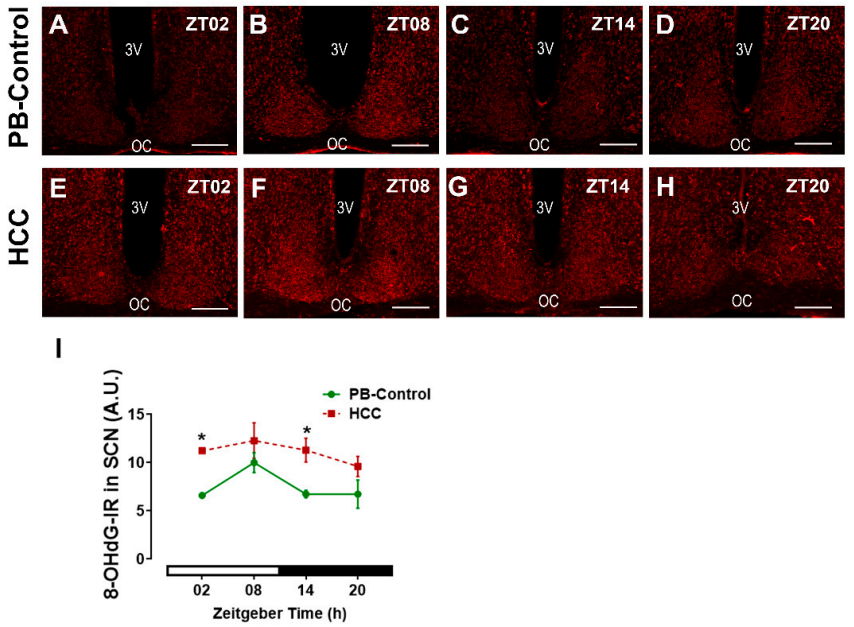

Figure 8. Oxidative stress marker 8-OHdG-immunoreaction in (IR) SCN. Representative fluorescent photomicrographs showing the immunoreaction (IR) of 8- hydroxydeoxyguanosine (8-OHdG) (red) in the SCN of (**A**–**D**) PB control mice and of (**E**–**H**) HCC-bearing mice. 3v: third ventricle. OC: optic chiasma. Scale bar = 100 µm. Mice were sacrificed at different time points at 6 h intervals starting at ZT02 = 2 h after the light was on. (**I**) Quantification of 8-OHdG-immunoreaction (IR) in arbitrary unit (A.U.) in the SCN. White and black bar indicates light/dark phase, respectively. Two-way ANOVA followed by Sidak's multiple comparisons test. Total of 12 mice per group, n = 3 mice at each time point. *: $p < 0.05$ between PB control and HCC.

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4. Discussion

There is increasing evidence of a bidirectional relationship between cancer and the circadian system. On the one hand, cancer patients suffer from sleep–wake disturbances and decreased activity, known as cancer-related fatigue [1]. On the other hand, circadian disruption is a potential risk factor for the development and progression of some cancers,

4. Discussion

There is increasing evidence of a bidirectional relationship between cancer and the circadian system. On the one hand, cancer patients suffer from sleep–wake disturbances and decreased activity, known as cancer-related fatigue [1]. On the other hand, circadian disruption is a potential risk factor for the development and progression of some cancers, including malignant breast and gastrointestinal tumors [49].

In this study, we provide novel evidence that HCC in mice leads to significant changes at the level of the key component of the circadian system, the SCN. In particular, we see changes in neuropeptides and the activity of neurons and glial cells, as well as increased oxidative stress in SCN.

We previously showed a significant reduction in the overall locomotor activity, especially during the dark phase, reduced the amplitude of the activity rhythm, and reduced the neuronal activity in SCN during the light phase [37]. In accordance, mice with chemically induced HCC showed alterations in the locomotor activity rhythm [50]. These tumor-related changes in mice are similar to the symptoms of patients with ovarian, breast, liver, and colorectal tumors [51,52], indicating an effect of tumorigenesis on the circadian system, such as reduced activity, increased fatigability, and activity–sleep rhythm abnormalities. Furthermore, the SCN becomes less sensitive to the environmental photic cues in patients with liver cirrhosis [53], which is considered a premalignant condition [54]. Thus, HCC mice and patients with malignant tumors seem to share common changes in the circadian system. On the one hand, the SCN clockwork ensures the anticipation of the light and dark phases. On the other hand, the SCN clockwork is adaptively modulated by the light/dark information through a projection from the retina.

In SCN, neuropeptides, particularly VIP and AVP, are important for synchronization with the light/dark cycle to sculpt critical features of the sleep–wake cycle and for determining the period of circadian rhythms [10,17,18,55–57]. Both VIP- and AVP-immunoreactive SCN neurons play an important role in mediating the circadian output rhythm to subordinate oscillators in the brain, such as the paraventricular nucleus of the hypothalamus (PVN) which controls rhythms in the autonomic nerve system [58], secretion of hormones such as melatonin [58], and glucocorticoids [10] as well as the sleep–wake cycle [10]. These appear to be species-dependent differences in whether the AVP- and VIP-immunoreaction exhibit a time-of-day-dependent rhythm [59–61]. However, there is no question that the VIP+ neurons in the mouse SCN are activated by light and play an essential role in light entrainment of locomotor activity rhythm [17,18]. Importantly, both the VIP- and the AVP-IR are higher in the SCN of HCC mice than in the PB control mice. This is consistent with VIP and its VPAC receptors being upregulated in various tumors, including HCC [62], pancreas [63], lung [51], breast cancers [64], and glioma [65]. This upregulation is probably due to inflammatory processes associated with cancer development [62]. Although we cannot conclude from immunohistochemistry whether the higher staining intensity of neuropeptides reflects higher or lower secretory activity, a change in staining intensity definitely reflects a change in functional status.

We could show earlier that in the brains of HCC mice, the expression of genes encoding for proinflammatory cytokines is increased [36], supporting this hypothesis. Moreover, we could show earlier that the serum corticosterone levels are increased in HCC mice [37]. However, we cannot differentiate whether this is due to the inflammatory response or the disruption of the output of the SCN to the PVN or the hypothalamic–pituitary–adrenal (HPA) axis. Nevertheless, glucocorticoids not only provide time information for peripheral clocks but also modulate the VIP and AVP systems in the SCN [66]. Thus, increased immunoreaction of VIP and AVP in the SCN of HCC mice observed in this study might be due to the inflammatory response mediated by cytokines and/or glucocorticoids. In addition, metabolic changes associated with HCC can have a neuroactive effect (also see below).

Orexin is a neuropeptide exclusively produced by a specific subset of neurons in the lateral hypothalamus, and its dysfunction is associated with increased sleepiness

and decreased activity [67,68]. Since there was no effect of HCC on orexin-IR in the lateral hypothalamus, a general impact on neuropeptide expression throughout the brain is unlikely.

VIP is important for robust rhythms in clock gene expression in the SCN and some peripheral organs [69] and for the entrainment of circadian rhythms to the light/dark cycle [10,18], presumably through CRE activation in the SCN [70], leading to c-Fos and clock gene expression [71]. However, chemogenetic activation of VIP+ SCN neurons attenuates the amplitude of circadian rhythms in the SCN, consistent with the concept that high levels of VIP lead to reduced synchronization between SCN cells [72]. Thus, the alteration of VIP and AVP systems in the SCN of HCC mice observed in this study might contribute to reduced precision in the alignment of activity to the light/dark cycle and the decreased amplitude in their activity rhythm previously reported in [37]. Consistently, the higher VIP-IR in HCC mice was associated with a decrease in c-Fos-IR (this study), similar to the decrease in pERK-IR [37] and an increase in Bmal1-IR (this study).

Bmal1 is essential for circadian rhythm generation [73] and the robustness of rhythmic activity in the light/dark cycle [12] In addition, Bmal1 is involved in the light-induced resetting of the SCN [13]. Bmal1-IR in the SCN shows no fluctuation depending on the time of day and is not affected by light [74]. Consistently, there was no effect of time on Bmal1-IR in the SCN of both groups. However, Bmal1 plays a critical role in circadian misalignment [75], and overexpression of Bmal1 leads to damping of SCN circadian rhythms [76]. Bmal1-IR was higher in the SCN of HCC mice compared to PB controls, suggesting a circadian misalignment and a mild overexpression. Thus, the lower levels of c-Fos and the higher levels of Bmal1 in the SCN of HCC mice might be due to the changes in the secretory activity of VIP+ neurons and might contribute to the reduced precision in the alignment of the activity rhythm to the light/dark cycle. Importantly, c-Fos is expressed in neurons only, while Bmal1 is also expressed in glia cells [14]. SCN astrocytes are important components of the rhythm-generating network, e.g., by regulating the balance between VIP and GABA [77]. They can initiate and maintain robust circadian activity rhythms and determine the circadian period [14,78]. Bmal1 might be involved in the regulation of the fine astrocytic processes that contribute to the chemical synapse [79]. The increased GFAP-IR in HCC mice indicates activation of astrocytes associated with the downregulation of their supportive function and upregulation of their proinflammatory function [80]. This might contribute to the alteration of neurotransmitter balance, reduced robustness of rhythmic locomotor activity, and also affect neurotransmission. Although there is no evidence for the involvement of microglia in the SCN circadian clock under physiological conditions [81], activation of proinflammatory microglia in SCN is associated with reduced rhythm amplitude in SCN slices [82]. The increased IBA-IR in HCC mice indicates activation of microglia accompanied by upregulation of their proinflammatory activity [83], which may contribute to impaired SCN function. Similarly, other peripheral malignant tumors, including breast cancer, trigger neuroinflammation and activation of glia cells [1,84] to secrete neurotoxic factors and chemokines and promote the recruitment of immune cells across the blood–brain barrier [80]. We hypothesize that cancer-related fatigue is related to impaired SCN function. Activation of astrocytes and microglia associated with liver diseases is also consistent with previous studies and may be a homeostatic response to protect brain tissue from higher levels of circulating cytokines and/or neuroactive/-toxic metabolites such as ammonia [85–88]. Both activated astrocytes and microglia can contribute to oxidative stress [89–91]. The increased IR for 8-OHdG-IR, a marker of DNA and RNA oxidative damage, in HCC mice, is consistent with higher oxidative/nitrosative stress and neuroinflammation in liver diseases [25,92,93]. Oxidation of DNA and RNA might contribute to transcriptional and posttranscriptional modulation of clock gene expression in the SCN of HCC mice [32]. This mechanism might be related to the effect of melatonin, which can act as a scavenger for radical oxygen species, on the consolidation of rhythmic locomotor activity in HCC mice [50]. In addition, our previous findings revealed that HCC triggered an increase and disruption in the rhythmic glucocorticoid in mice [37]. Similar

results were observed in HCC [30] and metastatic colorectal patients [94]. This chronic increase in glucocorticoids stimulates the stress response pathway via the HPA axis, which in turn stimulates the immune system to secrete various cytokines and chemokines, leading to neuroinflammation [95].

However, in the hippocampus of HCC mice, the expression of inflammatory cytokines and clock genes was increased, and the number of proliferating cells in the neurogenic niche was decreased, while the IR for c-Fos, pERK, GFAP, IBA, and 8-OHdG was not affected [36,37]. This suggests a brain region-specific difference in the response of neurons and glia cells to HCC and a modulation of clock gene transcription and adult neurogenesis by tumor-associated factors independent of oxidative stress.

Importantly, dysregulation of the endogenous circadian clock due to consuming alcohol, high-fat diets, shift work, or jet lag misaligns the liver's rhythmic metabolism and, thus, leads to liver pathologies such as HCC. A mice model with circadian malfunction due to chronic jet lag revealed more tendency to develop HCC in non-alcoholic fatty liver disease (NAFLD) due to disruption of the cell cycle components, including cell proliferation and apoptosis, and activation of oncogenic pathways such as WNT/β-catenin and TNFα-NF-κB in the liver [96].

This study has some limitations. The semi-quantitative measurements of the immunoreaction of relevant markers for neuronal and glial activity in the SCN have some limitations. While immunohistochemistry has the great advantage that it is restricted to the specific area under investigation and the signal is not "contaminated" by other brain areas as is the case in biochemical analyses of punch preparations, it cannot be determined whether the higher staining intensity of neuropeptides reflects higher secretory activity or storage of neuropeptides. This question can be solved only by additional invasive procedures such as microdialysis in future studies. It has been shown that HCC is more prevalent in males [97]; however, circadian rhythms are disrupted in both male and female HCC patients [30]. In addition, there is a sex-specific effect in a wide variety of circadian functions as well as in the response to chronodisruption [98]. Thus, it is still important to include HCC-bearing female subjects in future studies to identify sex-dependent differences in the response of the circadian system to HCC. It would also be of interest to detect the interaction between the development and progression of HCC and aging, which is correlated with dampened circadian rhythms, on the SCN function and the rhythmic behavior in further studies.

5. Conclusions

We propose a mechanism whereby cancer-related inflammation and/or neuroactive metabolites of liver dysfunction impair the circadian system at the level of the SCN. Our data suggest a double effect, on the one hand, with regard to neuronal activity and neuropeptides and, on the other hand, with regard to glial activity and oxidative stress. Both effects converge in a dysfunction of the molecular clockwork, which results in altered locomotor activity due to changed SCN rhythmic output. Our study contributes to the understanding of chronodisruption in cancer/liver diseases and could help develop corresponding therapeutic strategies to obtain better treatment outcomes and improve the quality of life of the patients.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines12102202/s1, Figure S1. Liver inspection during autopsy; Figure S2. Anatomical localization of SCN and lateral hypothalamus (LH); Figure S3. Neuropeptides immunoreaction in SCN.

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Data Availability Statement: The dataset supporting the conclusions of this article is available upon reasonable request to the corresponding author without restriction.

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Abbreviations

AVP: arginine vasopressin, CRF: cancer-related fatigue, DAB: 3,3′-diaminobenzidine, GFAP: fibrillary acidic protein, HCC: Hepatocellular carcinoma, IR: immunoreaction, 8-OHdG: 8-hydroxydeoxyguanosine, PB: Phenobarbital, PBS: phosphate buffered saline, PVN: paraventricular nucleus of the hypothalamus, RHT: retino-hypothalamic tract, SCN: suprachiasmatic nucleus, SEM: standard error of the mean, VIP: vasoactive intestinal peptide, ZT: Zeitgeber Time.

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3 Discussion

Cancer-related fatigue (CRF) and sleep-wake rhythm disturbance are common non-specific symptoms of HCC with a decreased level of locomotor activity (Qiu et al., 2020) and alteration in cortisol level (Hassan, Ali, Yassine, Sohn, Pfeffer, Jänicke, H. W. Korf, et al., 2021), which represent reliable readouts of the integrity of central rhythm generator in the SCN (Galani et al., 2001; Gong et al., 2015; Sallam et al., 2016), indicating that the HCC affects the SCN function.

Thus, in this study, we investigated the effect of HCC on the circadian system at the level of SCN. We found that HCC in mice leads to an alteration of neuropeptides, the essential circadian clock protein Bmal1, neuronal activity, in addition to activation of glia cells and increased oxidative stress in the SCN.

3.1 Altered SCN neuropeptides in HCC

VIP and AVP are essential neuropeptides expressed by SCN core and shell neurons, respectively, that play a crucial role in circadian system integrity. VIP-positive neurons are the main retinorecipient cells in SCN core and are particularly important for the entrainment of circadian rhythms to the light/dark cycle and synchronization of SCN neurons (Jones et al., 2018; Ono et al., 2021; Vosko et al., 2015), while AVP is important for rhythms (Tousson & Meissl, 2004; Yamaguchi et al., 2003)(Tousson & Meissl, 2004; Yamaguchi et al., 2003).

In our study, there was an increased VIP and AVP Immunoreaction (IR) in HCC mice at dark/activity phase, suggesting that HCC affects these neuropeptides in SCN. In line with our findings, previous studies showed that various tumors may induce an upregulation of VIP and VIP receptors, including breast, lung and prostate cancer (Hara et al., 2019; Moody et al., 2016), suggesting that cancer may trigger VIP upregulation as a part of an inflammatory process. This hypothesis is supported by the release of VIP from T-lymphocytes detected in vitro (Martinez et al., 1999). An upregulation of VIP, as a modulator in inflammatory reactions, may aim to prevent the HCC progression through apoptosis, via a cAMP-mediated signaling pathway (Hara et al., 2019).

Furthermore, cancer cells of various origins can independently express vasopressin, for example small cell lung cancer (North et al., 2000) and elevated AVP mRNA levels were also detected in mice with prostate cancer(Heidman et al., 2022).

In addition, our previous data showed increased corticosterone levels due to HCC in mice (Hassan, Ali, Yassine, Sohn, Pfeffer, Jänicke, H. W. Korf, et al., 2021).

Importantly, glucocorticoids such as cortisol are not only a synchronizer for the periphery, but also a modulator for the neuropeptides (Han et al., 2012)(Harlan et al., 1988).
In sum, the tumor-induced inflammatory reaction and increased corticosterone levels caused by the cancer may upregulate neuropeptide IR in SCN.
As VIP and AVP are important for the synchronization of circadian rhythms, a change in their level may lead to a dysregulation of the sleep-wake cycles and spontaneous locomotor activity levels (Su et al., 2015). In line with this hypothesis, an increased level of VIP leads to reduced synchrony between the SCN cells (An et al., 2013).
This leads to the assumption that changes in the neuropeptides in the active/dark phase, triggered by the HCC, may reduce the SCN synchrony and thus, affect the rhythmic locomotor activity levels and dampened amplitude of the circadian rhythm (Hassan, Ali, Yassine, Sohn, Pfeffer, Jänicke, H. W. Korf, et al., 2021).

3.2 Reduced SCN neuronal activity in HCC

Light-induced signal transduction in the SCN is demonstrated by activation of p-ERK/MAPK signaling pathway leading to c-Fos expression (Smith et al., 2015b). Thus, c-Fos and p-ERK can be used as a marker for rhythmic SCN neuronal activity.
SCN neuronal activity, as indicated by p-ERK (Hassan, Ali, et al., 2021) and c-Fos (this study) immunoreaction, showed time-of-day-dependent changes in HCC mice, similar to control mice, however, with a decreased amplitude, indicating that HCC dampens the SCN neuronal activity. This is reminiscent of liver cirrhosis patients, which are less sensitive to environmental photic information (Costa et al., 2023), suggesting that liver chronic pathologies can lead to a reduced neuronal activity in the SCN. As synchronization of SCN neurons to photic signals is mediated by VIP, the alteration of VIP levels may influence the neuronal activity in SCN in HCC mice.

3.3 Altered SCN molecular clock in HCC

Bmal1 is an essential clock protein which plays an important role in the readjustment of the SCN cells to light-induced influences (Pfeffer et al., 2009). Bmal1 immunoreaction does not show a time of day-dependent rhythm (Von Gall et al., 2003), which agrees with our findings in the control mice group. Importantly, overexpression of Bmal1 dampens circadian rhythms (Ikeda & Ikeda, 2014) and may lead to circadian misalignment (Zuo et

al., 2024). In this study, there was an increase in Bmal1-IR in HCC-bearing mice. This is also in agreement with our previous study that showed that HCC affects the expression of circadian genes in the hippocampus (Yassine et al., 2023). Other findings suggested that cancer may impact the molecular clockwork at the SCN levels. For instance, subcutaneous implantation of B16 melanoma cells affected clock genes (Bmal1 and Cry1) expression in the SCN, which was associated with a reduced nocturnal locomotor activity and dysregulated corticosterone levels (Aiello et al., 2022). Furthermore, clock genes may be affected by tumor independently on their role of the circadian clock. For example, Bmal1 was upregulated in association with various tumors, primarily, due to errors in cell cycle controls and DNA repair mechanisms (Verlande & Masri, 2019) and Cry2, which inhibits the cell cycle, is downregulated in adeno- and squamous cell carcinoma of the lung and correlated with tumor progression (Yang et al., 2019).

We assume that the decreased number of active neurons, as indicated by c-Fos-positive cells, and the increased Bmal1-IR in the SCN in HCC mice may be a result of altered VIP secretion and may be accountant for the misalignment of the activity rhythm to the light/dark cycle.

3.4 Increased SCN glial activity and oxidative stress in HCC

SCN astrocytes play an important role for circadian functions (Nakamura, 2010). Importantly, astrocytes in the SCN are an important component in regulating the balance between VIP and GABA (Barca-Mayo et al., 2017) and can maintain a functional clockwork and rhythmic locomotor behaviour independent on neurons (Brancaccio et al., 2019b). The role of microglia in circadian rhythm generation under physiological conditions is not fully clear.

Both astrocytes and microglial cells release and respond to cytokines (Tso et al., 2017) and their activation reflects an inflammatory process in the respective tissue (Lawrence et al., 2023a). In our study, we observed a higher intensity of GFAP immunoreaction, which is a marker for reactive astrocytes (Liddelow & Barres, 2017), in HCC mice during the dark/active phase. Similarly, we noticed higher IBA1 immunoreaction, which is a marker for microglia cells (Lier et al., 2021), during the dark/active phase in HCC compared to the control group. The upregulation of IBA1 is strongly associated with increased proinflammatory activity of the cells (Ito et al., 1998). This indicates an activation of the glial cells in SCN.

Importantly, other malignant tumors, such as breast cancer, also led to the activation of glial cells (Lawther et al., 2022b). Activated glial cells can release neurotoxic substances and chemokines, which recruit additional immune cells via the blood-brain barrier (Lawrence et al., 2023b), The proinflammatory microglia in the SCN, reduced the rhythm amplitude (Honzlová et al., 2023b). All these factors may affect the integrity of brain functions including the circadian rhythms and may be correlated to CRF.

Next, we investigated the oxidative stress levels in SCN using 8-OHdG (Kasai et al., 1997), which is closely related to neuroinflammation indicated by astrocyte and microglial activation (Chen et al., 2020). 8-OHdG-IR was increased at all time points in SCN of HCC mice, indicating that HCC could triggers oxidative stress in the brain and may impair the function of the SCN. Previous research empathised that higher oxidative stress due to liver diseases can be associated with neuroinflammation (Görg et al., 2010; Simicic et al., 2022; Valavanidis et al., 2009). In addition, the expression of clock genes can be altered due to oxidative stress via increased DNA and ribonuleic acid (RNA) oxidation (Y. M. Lin et al., 2008).

We assume that the HCC induced neuroinflammation including glial activation and increased oxidative stress, which in turn may impact the neuronal activity and the molecular clock in SCN and, thus, may contribute to the misalignment of the activity rhythm to the light/dark cycle.

3.5 Conclusion

Our results suggest that HCC has multiple effects: firstly, alterations in neuropeptide expression and neuronal activity, and secondly, altered glial function and increased oxidative stress. These effects could synergistically and negatively impact the rhythmic SCN function, ultimately leading to disruptions throughout the circadian system.

This study expands the current understanding of chronodisruption in the context of cancer and liver disease and can contribute to the development of targeted therapeutic strategies aimed at improving treatment effectiveness and patients' quality of life.

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5 Appendix

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