Setting Dopamine's Rhythm: The Role of The Habenula as a Pacemaker for Rhythmic Dopamine

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Abstract For PhD

Setting Dopamine's Rhythm: The Role of the Habenula as a Pacemaker for Rhythmic Dopamine

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Dopamine (DA) plays a crucial role in regulating a variety of behaviours, including motor function and reward. While the presence of DA is essential, its rhythmic release into regions such as the striatum is equally critical for maintaining normal physiological functions. This thesis investigates the hypothesis that the lateral habenula (LHb) serves as a pacemaker for rhythmic DA release, independently of the suprachiasmatic nucleus (SCN). The habenula's circadian pacemaker properties and its projections to the substantia nigra (SN) and ventral tegmental area (VTA) suggest it could regulate DA rhythms.

To test this hypothesis, we performed a targeted knockout of *Bmal1* in the LHb of male and female mice and assessed the impact on motor and alcohol consumption behaviours. Our findings indicate significant disruptions in motor function, DA levels, and gene expression in the SN and dorsal striatum (DS) following LHb *Bmal1* knockout. Specifically, we observed altered rhythms in clock genes and DA-related genes, supporting the LHb's role in regulating DA rhythms.

We further explored therapeutic strategies to mitigate the observed motor deficits using pharmacological (quinpirole) and non-pharmacological (running wheels) interventions. Both interventions improved motor performance, with notable sex-specific responses, highlighting the complexity of motor regulation by circadian mechanisms. However, neither intervention fully restored normal DA levels, suggesting involvement of additional pathways, such as the serotonergic system.

Additionally, we found sexually dimorphic effects on alcohol consumption behaviours following LHb *Bmal1* knockout. Male mice showed increased voluntary alcohol intake and binge drinking, while female mice displayed reduced consumption under specific conditions. These results underscore the interaction between the LHb, DA, and serotonin systems in regulating alcohol-related behaviours.

In conclusion, this thesis demonstrates that the LHb acts as a pacemaker for rhythmic dopamine release, influencing both motor and reward behaviours. The findings provide new insights into the

complex interplay between circadian rhythms and the dopaminergic system, with potential implications for the LHb as a novel site for therapeutic interventions targeting both systems. Understanding the role of the LHb in setting DA's rhythms offers a novel perspective on neuronal functioning and circadian biology.

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Contribution of authors

Chapter 1: Co-authors include Nadav Baharav, Heng Jiang, Amanda Szubinski, Shimon Amir, and Konrad Schoettner. K.S, S.A., and C.G. conceived and designed the study; C.G., N.B., H.J., and A.S. performed the experiments; C.G., K.S., and S.A. analyzed and interpreted the data, and wrote the manuscript; S.A. and K.S. Supervision.

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All authors reviewed the final manuscripts and approved the contents.

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List of Abbreviations

5-HT: Serotonin

AAV: Adeno-associated virus

ANOVA: Analysis of variance

AUD: Alcohol use disorder

BMAL1, ***Bmal1***: Brain and muscle arnt-like, PROTEIN or *gene*

CLOCK, ***Clock***: Circadian locomotor output cycles kaput, PROTEIN or *gene*

CRE: cAMP/Ca2+ responsive element

CRY, ***Cry****:* Cryptochrome, PROTEIN or *gene*

Ctrl: Control mouse

D1R: Dopamine receptor 1

D2R: Dopamine receptor 2

DA: Dopamine

DS: Dorsal striatum

DID: Drinking in the dark

EPM: Elevated plus maze

GFP: Green fluorescent protein

HBT: Horizontal bar test

IAE: Intermittent alcohol exposure two-bottle choice test

KO: Knockout mouse

LD: Light/ dark

LL: Constant light

LHb: Lateral habenula

MAOA, Maoa: Monoamine oxidase A, PROTEIN or *gene*

MSN: Medium spiny neurons

NAc: Nucleus accumbens

OFT: Open-field test

PD: Parkinson's disease

PER, ***Per***: Period, PROTEIN or *gene*

REV-ERBα, ***Rev-Erbα***: REV-ERB, PROTEIN or *gene*

RMTg: Rostral medial tegmental area

RORα, ***Rorα***: RAR-related orphan receptor, PROTEIN or *gene*

SCN: Suprachiasmatic Nucleus

SN: Substantia nigra

SPT: Sucrose preference test

TH, *TH*: Tyrosine hydroxylase, PROTEIN or *gene*

TST: Tail suspension test

TTFL: Transcriptional/ translational feedback loop

VTA: Ventral tegmental area

WT: Wild-type mouse

ZT: Zeitgeber time

Introduction

Dopamine (DA), a catecholamine neurotransmitter, is central to the functioning of the mammalian brain, orchestrating behaviours such as learning, motor control, and reward. DA's multifaceted functions encompass complex mechanisms crucial for both physical and mental health. Dysregulation of DA signaling can lead to significant neurological and psychiatric disorders, most notably Parkinson's disease and addiction.

Synthesized primarily in the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) (Björklund & Dunnett, 2007), DA projects to various brain regions, including the striatum, via the nigrostriatal (dorsal striatum, DS) and mesolimbic (nucleus accumbens, NAc) pathways. Within the striatum, two main pathways—the direct and indirect—mediate opposing actions through DA 1 (D1) and DA 2 (D2) receptors, respectively. D1 receptors are associated with the direct pathway and stimulatory "go" actions, while D2 receptors are linked to the indirect pathway and inhibitory "no go" actions (Albin et al., 1989; Ramesh & Arachchige, 2023). Balance between the direct and indirect pathways, and thus a balance in levels of D1 and D2 expressing neurons, is essential for typical striatal efficiency (Chen et al., 2013; Dickinson & Elvevåg, 2009; Vijayraghavan et al., 2007; Williams & Castner, 2006).

DA's role in motor control is illustrated in Parkinson's disease (PD), a neurodegenerative disorder affecting over 100,000 Canadians (Rizek et al., 2016). Characterized by tremors, rigidity, and bradykinesia, PD is directly linked to the degeneration of DA-producing neurons in the SN. This leads to an imbalance between the direct and indirect pathways, specifically an overactivity of the “no go” (indirect) pathway that leads to these stereotypical motor symptoms (Dauer & Przedborski, 2003; Ramesh & Arachchige, 2023).

Equally significant is DA's role in addiction. In Canada, it is estimated that 21% of the population will meet the criteria for addiction at some point in their lives (Health Canada, 2023) . Addictive substances stimulate DA release in the brain, creating intense feelings of pleasure which can lead to dependence (Wise & Jordan, 2021). The NAc is primarily responsible for these pleasurable effects, while the DS is implicated in the development of habitual, dependent drug use (Everitt & Robbins, 2016; Salamone & Correa, 2012). While an imbalance between the direct and indirect pathways has been thought to mediate addictive behaviours, the direction of this effect appears to be drug- and region- dependent (Edwards et al., 2007; Trifilieff & Martinez, 2014; Yager et al., 2015).

Notably, PD and addiction share another important common link: circadian rhythms. Circadian rhythms are the 24-hour cycles in physiology and behaviour generated by an internal circadian clock and regulated by the external environment. Examples include the sleep-wake cycle, feeding, drinking, and locomotor activity. Disruptions in circadian rhythms can lead to adverse health consequences. For example, up to 80% of individuals with PD experience sleep disturbances, and drug intake has been found to disrupt daily rhythms (Honma & Honma, 2009; Kosobud et al., 2007). Furthermore, shift workers, who often suffer from circadian misalignment, exhibit an increased prevalence of both PD and addiction (Doyle et al., 2015; Kirkpatrick et al., 2009).

The link between DAergic signaling and circadian rhythms supports a bidirectional relationship, wherein disruptions in one system can significantly impact the other. For example, Hood and colleagues demonstrated that loss of DA disrupts circadian rhythms of clock gene expression in the striatum, a region essential for both motor and addictive behaviours (Hood et al., 2010). Conversely, DA levels exhibit rhythmic properties under the control of the circadian clock, with higher concentrations observed at night in rodents (Domínguez-López et al., 2014; Ferris et al., 2014; Hood et al., 2010).

Despite ample experimental and clinical evidence of a bidirectional interaction between the DAergic and circadian systems, knowledge as to the mechanisms that control this interaction and the behavioural consequences that result from their dysregulation, remains limited. The research presented in this thesis aims to elucidate the role of the circadian clock in striatal DA signaling and associated motor and addiction related behaviours. Specifically, it aims to study the influence of a circadian clock mechanism in the lateral habenula (LHb), a major regulator of DA synthesis in the SN and VTA, on striatal DA functioning. Moreover, we aim to characterize the behavioural consequences resulting from dysregulation of this clock mechanism. Summarized below encompasses the current knowledge pertinent to the present investigation.

The Molecular Clock: The Inner Workings of Circadian Rhythms

The discovery of the internal mechanisms of the mammalian clock was made possible by screening for mutants with altered circadian systems (Hastings et al., 2019). This led to the identification of "core clock genes," including Period (Per) in Drosophila and Clock in mice (Hardin et al., 1990; Konopka & Benzer, 1971; Vitaterna et al., 1994). The molecular basis of the

mammalian circadian system is now understood to be mediated through interlocked transcriptional/translational feedback loops (TTFL) (King & Takahashi, 2000). Here, *Clock* (circadian locomotor output cycles kaput) and *Bmal1* (Brain and Muscle ARNT-like 1) acts as the positive arms of this loop. These basic helix-loop-helix transcription factors heterodimerize via PAS domains to bind to DNA at Enhancer boxes (E-boxes) in the promoter regions of *Per* (Period) and *Cry* (Cryptochrome), forming the negative arm of this loop. PER and CRY proteins in turn inhibit BMAL1/CLOCK transcriptional activity, thereby suppressing their own expression in a process repeated every 24 hours. Additionally, *RORα* (RAR-related orphan receptor alpha) and *Rev-erbα* (NR1D1) bind to ROR elements in the *Bmal1* promoter region, ensuring that *Bmal1* expression remains in anti-phase with *Per* and *Cry*, further stabilizing the clock mechanisms. *Bmal1* is crucial as it is the only clock gene absolutely required for circadian function; its removal results in the complete loss of circadian rhythms, unlike the mere disruption seen with the removal of any other core clock gene (Bunger et al., 2000; Haque et al., 2019).

The Central Pacemaker: An Orchestra of Clocks

In mammals, circadian rhythms are driven by a central pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Dibner et al. 2010; Hastings et al. 2019) and regulated by subordinate clocks distributed throughout the brain and body. Connections between the SCN and other brain structures have been revealed through the use of antero- and retrograde tracers, showing that SCN efferents terminate in various brain sites, but, not to every clock in the brain and body (Dibner et al., 2009). As such, there is much discussion as to what extent the SCN clock is needed to set rhythms in these peripheral clocks.

There are two prevailing theories for how the SCN interacts with peripheral clocks. One theory posits a "hierarchical" model, a tiered system in which the SCN alone synchronizes peripheral clocks to the external environment. The second, the "orchestra" model, proposes that the SCN is more like the conductor in an orchestra, sending signals to ensure rhythmicity throughout the brain and body, but that peripheral clocks can also entrain to various internal and external zeitgebers on their own (Davidson et al., 2003; de Assis & Oster, 2021a). Ample evidence supports the second model as being the one governing the relationship between the central and peripheral clocks, as exemplified in the liver clock, where food acts as the primary time cue (Chavan et al., 2016; Feillet et al., 2006).

Research into peripheral clocks and how they function is vital to our understanding of circadian functioning, as these clocks are responsible for maintaining the rhythmicity of tissue-specific functions (Balsalobre et al., 1998; Nagoshi et al., 2004). Initially, it was thought that only the SCN clock could be self-sustained, with other clocks' rhythmic functioning dampening rapidly without SCN input. However, Yoo demonstrated that some peripheral clocks continue to oscillate without a direct link with the SCN (Yoo et al. 2004). This suggests that many extra-SCN brain regions and peripheral organs have intrinsic oscillating properties whose rhythms are not controlled exclusively by the SCN, but also by other external and internal timing signals (Guilding & Piggins, 2007a; S.-H. Yoo et al., 2004).

Zeitgebers: Environmental cues

To align our internal clocks with the external world, circadian systems rely on timing cues known as zeitgebers (Aschoff, 1960; Dibner et al., 2009). The primary zeitgeber of the SCN is light, perceived by intrinsically photosensitive retinal ganglion cells (ipRGCs) that express the photopigment melanopsin, and communicate to the SCN via the retinohypothalamic tract (Berson et al., 2002; Dibner et al., 2009; Hastings et al., 2019). A recently identified pathway from these ipRGCs to the peri-habenular nucleus (Fernandez et al., 2018) and a pathway from the retinorecipient intergeniculate nucleus to the LHb (Huang et al., 2019) have been brought to light. This therefore recognizes that light information, the primary time-cue in the brain, is not only processed by the SCN clock, but rather any clock with retinal input and the retina clock itself have the capacity to entrain to light cycles independently.

While light is the primary zeitgeber, other non-photic cues such as food (Feillet et al., 2006; Mendoza, 2007), exercise (Hughes & Piggins, 2012), and socialization (Mistlberger & Skene, 2004), also play significant roles in synchronizing rhythms. This brings us back to dopamine (DA), which we know has a reciprocal interaction with the circadian system and appears capable of entraining clocks in the brain – therefore acting as an essential zeitgeber (Steele & Mistlberger, 2015). Early research showed DA's role in entraining circadian rhythms in fetal rats, with D1 receptor stimulation in the SCN altering only pups' activity rhythms (Viswanathan et al., 1994; Weaver et al., 1992). Rhythmic DA in the retina plays a role in process of transmitting light signals to the SCN clock (Gooley et al., 2001; Hattar et al., 2006; Popova, 2014; Prigge et al., 2016). Specifically, in the retina, D1 receptors are responsible for

entraining *Per2* rhythms (Ruan et al., 2008) and D2 receptors entrain *Per1* rhythms (Yujnovsky et al., 2006). In the striatum, DAergic input is required for rhythmic expression of clock genes (Hood et al., 2010; Imbesi et al., 2009), and contrary to the retina, D2 rhythms moderate striatal PER2 expression (Hood et al., 2010). 6-OHDA lesions disrupt activity rhythms and total activity levels under constant darkness, supporting that DA signaling is required to maintain rhythmicity (Gravotta et al., 2011). Moreover, D1 receptor dysfunction in rodents was found to diminish the speed of entrainment (Grippo & Güler, 2019) and impairs food anticipatory activity (Gallardo et al., 2014). Simply put, typical DA signalling is essential for the maintenance of circadian rhythms in many regions throughout the brain.

The opposite is true as well. The midbrain DA nuclei require the circadian clock for proper functioning, and this finding has been supported through mathematical modeling assessing available data (Kim & Reed, 2021). Tyrosine hydroxylase (*Th*), the rate-limiting enzyme in DA synthesis, shows diurnal variations in the substantia nigra (SN), ventral tegmental area (VTA), and the dorsal striatum (DS) (Ferris et al., 2014; Owasoyo et al., 1979; Paulson & Robinson, 1995; Webb et al., 2009). These *Th* levels are highest at night when *Rev-Erbα* levels are lowest (Chung et al., 2014) and knocking out *Rev-Erbα* increases *TH* levels, with loss of daily variations. *Nurr1,* a transcription factor involved in the regulation of midbrain DA neurons, competes with *Rev-Erbα* to promote *TH* expression, and *Nurr1* knockout mice show altered activity rhythms (Partington et al., 2021). A global KO of *Bmal1* resulted in the loss of SN Th positive neurons (Kanan et al., 2024) and a *Bmal1* deletion in a Parkinson's mouse model (MPTP) exacerbated motor deficits and loss of *Th* positive neurons (Liu et al., 2020). Monoamine oxidase A (*Maoa*), a DA metabolizing enzyme, demonstrates a circadian pattern of expression in the VTA (Hampp et al., 2008). In *Per2* mutant mice, *Maoa* rhythms are blunted, leading to elevated striatal DA levels. Additionally, DA transporter (DAT) governs striatal extracellular DA levels, responsible for maintaining robust rhythms in the striatum (Ferris et al., 2014) which are essential for rhythmic *Per2* expression (Hood et al., 2010). To summarize, the clock has a strong influence on the functioning of enzymes responsible for DA synthesis and metabolism.

The bidirectional relationship between the clock and the DAergic system can be seen in individuals with Parkinson's disease (PD). Nighttime *Bmal1* levels are blunted in PD patients comparison to controls (Cai et al., 2010; Ding et al., 2011; Hunt et al., 2022) and these blunted

levels were found in both patients taking DA agonists (L-DOPA) and non-medicated patients. However, this study looked at *Bmal1* levels after L-DOPA treatment. We know from animal models that DA exerts the most direct impacts on Period genes, while *Bmal1* appears to directly impacts DA (Kanan et al., 2024). As such, L-DOPA as a DA agonist would have most likely impacted the levels of different Period genes, if any impact would be found. Moreover, *Bmal1* levels were shown to be negatively correlated with PD severity (Cai et al., 2010). Heightened levels of PER2 and REV-ERBα were found in the early morning, with blunted levels of BMAL1 at night in PD samples (Breen et al., 2014; Hunt et al., 2022). Together, this shows a strong pattern of clock gene dysfunction in those with PD.

Likewise, addiction studies find that mice lacking *Clock* or *Per2* genes were more strongly rewarded by cocaine administration (Abarca et al., 2002; Mcclung et al., 2005). Conversely, mice with *Per1* knockdown experience no signs of reward after cocaine injection (Abarca et al., 2002). A whole striatal *Bmal1* knockout led to increased alcohol drinking in males, but attenuated drinking in females (de Zavalia, 2021). Whereas a NAc specific *Bmal1* knockout led to increased alcohol drinking in both males and females (Hererra et al., 2022). *Bmal1* levels have been linked to alcohol consumption, and a *Bmal1* polymorphism has been associated with a risk for alcohol use disorder (Banach et al., 2018; Kovanen et al., 2010). As well, levels of *Bmal1*, *Per2*, *Cry2*, and *Rev-erb* genes were increased in hippocampus of those with substance use disorder (Valeri et al., 2022). Together this shows that the clock can impact drug taking behaviour and, in turn, the use of drugs impacts the clock.

Notably, while SCN lesions blunt DA rhythms in the striatum, it does not abolish them, thereby suggesting the presence of more than one DAergic pacemaker (Sleipness et al., 2007). Thus, while historically circadian research has focused on the SCN and its response to light inputs, it is evident that a comprehensive understanding of the interaction between DA and the circadian system requires a broader examination of peripheral oscillators and non-photic zeitgebers. As such, the current thesis focuses on the nigrostriatal pathway and its habenula inputs. These regions not only receive and respond to circadian signals but also play pivotal roles in motor control and reward processing.

The Striatum: A Critical Hub

The striatum, the primary input structure of the basal ganglia, stands out due to the

circadian clock it houses and its critical position in motor control, learning, cognition, and habit formation (Haber, 2014; Imbesi et al., 2009; Prager & Plotkin, 2019). Two major subregions of the striatum, the caudate putamen/dorsal striatum (CP/DS) and the nucleus accumbens (NAc), receive DAergic projections from the substantia nigra (SN) and the ventral tegmental area (VTA), respectively. As discussed previously, the medium spiny neurons of the striatum can be divided into two functionally distinct pathways, the direct (D1) and indirect (D2) pathways (Gerfen & Surmeier, 2011). D1 neurons of the direct pathway are responsible for "go" actions, whereas D2 neurons in the indirect pathways are associated with inhibitory "no go" actions.

While DA acts as a zeitgeber in a number of brain regions, its impact on the striatum is of particular interest. Application of a D1 agonist in cultured striatal neurons induced stimulatory responses on *Clock* and *Per1,* expression, whereas treatment with a D2 agonist had an inhibitory effect on the same clock genes (Imbesi et al. 2009). Additionally, DA induces the expression of PER2 in the DS, via the indirect pathway, supporting a crucial function of DA in the striatal clock (Hood et al. 2010). Conversely, parts of the DAergic signaling pathway in the striatum are clock-controlled (Ikeda et al., 2013). After a striatal specific *Bmal1* knockout, animals demonstrated decreased motor functioning, and showed an attenuated response to the D1 receptor agonist SKF, with decreased mobility in the open-field test (Schoettner et al., 2022). As such, it appears that rhythmic DAergic inputs to the striatum are essential for both the clock and typical behavioural functioning. The primary DAergic input structure to the DS is the SN.

The Substantia Nigra: Dopamine's Production

Directly projecting to the striatum, the substantia nigra (SN) has a critical role in modulating motor movement and reward functions as part of the basal ganglia circuitry (Guatteo et al., 2009; Haber, 2014). The SN can be divided into the pars compacta region (SNc), containing DAergic neurons, and the reticulata region (SNr), containing GABAergic interneurons (Guatteo et al. 2009). Research linking the SN to motor functioning began with evidence of its role in Parkinson's disease (PD) (Haber, 2014). Typical SN functioning, and thus normal DAergic synthesis and release, is critically influenced by the circadian clock. Long-term constant light exposure reduces tyrosine hydroxylase (TH)-positive neurons in the SN and decreases DA in the striatum (Romeo et al., 2013, 2017). *TH* is directly suppressed by *Clock* (McClung et al. 2005), and daily variations in *TH* levels were absent in *Rev-erbα* KO mice,

likely due to findings of abnormally high *TH* levels in these mice (Chung et al. 2014). *Maoa* shows circadian patterns in the VTA and is controlled by *Per2* (Hampp et al. 2008). As such, while DA has the strongest impact on the clock in the striatum, it is in the SN where the clock appears to have the greatest impact on DAergic functioning.

Notably, while both the DS and the SN possess circadian clocks, these are rapidly dampening oscillators that require signals from an external pacemaker. It is clear from the above literature that DA is rhythmic in its synthesis and release, most likely due to the clock regulation in the SN. Nevertheless, the mechanism that acts as a pacemaker for SN DAergic rhythms is unknown. There is no direct link from the SCN to the SN, nor any clear retinal pathways, suggesting that clocks elsewhere in the brain must be involved. Clocks, such as the one in the habenula.

The Habenula: A Key Pacemaker

The Habenula emerges as a critical structure in the circadian system. A small pair of nuclei in the epithalamus, the habenula is divided into the lateral and medial portions (Hikosaka et al. 2008). While both the medial habenula (MHb) and the lateral habenula (LHb) primarily receive their inputs from the frontal cortex, thalamus, and globus pallidus internus (Baker et al. 2016), they differ in their efferent pathways. The MHb mainly projects to the interpeduncular nucleus and the pineal gland (Carlson et al., 2001; Herkenham & Nauta, 1979; Rønnekleiv & Møller, 1979; Viswanath et al., 2014). Conversely, LHb projections directly and indirectly innervate the VTA, SN, and dorsal raphe (Christoph et al., 1986; Ji & Shepard, 2007; Langel et al., 2018; Matsumoto & Hikosaka, 2007). As such, due to the LHb's connections to the regions responsible for rhythmic DA synthesis and release, the present thesis will focus on this region of the habenula. Known as the negative reward center, LHb glutamatergic neurons activate in response to aversive stimuli and inhibit in response to reward (Ji & Shepard 2007; Matsumoto & Hikosaka 2007; Salaberry & Mendoza, 2016). The LHb is especially interesting because of the circadian clock it houses.

To be qualified as circadian pacemaker, a structure must meet three criteria. First, a structure must demonstrate rhythmic activity. LHb neurons show a higher firing rate during the day (inactive phase) in rodents (Sakhi, Belle, et al., 2014; H. Zhao & Rusak, 2005). Additionally, C-Fos expression in the LHb was correlated with heightened activity at night (Chastrette et al.,

1991; Paul et al., 2011). As such, the LHb displays clear evidence of rhythmic activity.

Next, the structure must possess rhythmic molecular clockwork. The expression of circadian clock genes in the LHb was first shown in a rat model, however due to the singular time point for tissue collections, no knowledge as to the rhythmic expression of these genes could be gained (Shieh, 2003). Rhythmic activity was first demonstrated in the mouse LHb ex-vivo, using PER2: LUC mice, indicating the presence of circadian oscillations in the LHb, where rhythms persisted for up 72h, even after the addition of sodium channel blockers (Guilding et al., 2010a, 2013). Interestingly, similar to functioning in the SCN, the period of PER2:LUC rhythms in the LHb can be lengthened by the Afterhours mutation, indicating conserved functioning between the structures (Guilding et al., 2013). This was followed by studies showing that *Per1*/*2* and *Bmal1* in the LHb rhythmically oscillate (Christiansen et al., 2016; Sakhi, Wegner, et al., 2014; B. Zhang et al., 2016a), along with *Per1-3*, *Dbp*, and *Nr1d1* levels (H. Yoo et al., 2022). To summarize, ample evidence of rhythmic clock gene expression has been found in the LHb, therefore clearly indicating the second criteria for pacemaker classification is satisfied.

The final criterion states that the structure must have the capacity for self-sustained oscillations (Guilding and Piggins, 2007). Salaberry and colleagues demonstrated that PER2:LUC rhythms persist despite SCN lesions and that clock genes persisted in their rhythmic expression under constant lighting conditions (both constant darkness and constant light) (Salaberry et al., 2019). However, the authors observed an uncoupling between the medial and lateral portions of the habenula when the SCN was lesioned, and rats were kept under constant darkness. This therefore suggests that while individual regions of the habenula may be capable of self-sustained rhythmicity, either light information or SCN timing signals are required for the habenula to oscillate cohesively.

Moreover, the habenula has the capacity for photic entrainment due to direct and indirect retinal projections. Retrograde tracers demonstrated a direct retinal projection to the LHb in rats (Qu et al., 1996). In mice retinal cells expressing the photopigment melanopsin project their axons to the LHb (Hattar et al., 2006). There are also indirect connections through the ventral lateral geniculate and the intergeniculate leaflet (L. Huang et al., 2019). Viral tracing shows that these structures receive direct input from retinal ganglion cells, and optogenetic studies reveals that activating this pathway causes and inhibitory response in LHb neurons. Whether this connection is only indirect, or also direct is under much debate and requires further examination,

but it is clear that LHb cells respond to light stimulation (Huang et al., 2019; Sakhi, Wegner, et al., 2014; Zhao & Rusak, 2005). Varying levels of light intensities were shown to increase mouse LHb neuronal activity (Sakhi et al., 2014b). In rats, light can stimulate or inhibit different groups of LHb cells in vivo (Zhao and Rusak, 2005). Even a short burst of light at night can increase c-Fos expression in the LHb (Shuboni et al., 2015). However, some studies have found that there appears to be a timing delay between light stimulation and LHb reaction that appears long enough to suggest that input is indirectly projected and thereby sheds doubts on a direct photic stimulation (Sakhi et al., 2014). Together, this research supports that the LHb has the capacity for self-sustained oscillations – at least to some degree. Due to the need for either light information or a functional SCN clock to maintain coupling between the regions and the potential lack of direct photic input, it would be reasonable to classify the LHb clock as a semi-autonomous oscillator.

Currently, the influence of the circadian clock housed in the habenula is poorly understood. However, based on the LHb's control over the regions responsible for DAergic synthesis and release, as well as its classification as a circadian oscillator, we hypothesize that the LHb acts as the pacemaker for daily DAergic rhythms.

The Present Thesis

The experiments presented in this thesis aim to unravel the question: Is the LHb setting the pace for rhythmic DA expression, and, if so, what is the importance of these rhythms? To explore these questions, we employed multiple experimental approaches. *Bmal1* was chosen here as its removal abolishes rhythms, rather than merely causing dysregulation (Bunger et al., 2000). The experiments are therefore linked by the use of a LHb-specific *Bmal1* knockout (KO). These mice are genetically engineered with loxP sequences flanking the *Bmal1* gene on exon 8 (Storch et al., 2007). Cre virus injected directly into the LHb during stereotaxic surgeries targets these loxP sites, creating a stop sequence and silencing the gene. As we hypothesize that our manipulation will impact DAergic functioning, behavioural assessments of these KO mice will primarily focus on motor functioning and addiction-like behaviours. Given the essential roles of the substantia nigra and dorsal striatum in DAergic functioning and in these behaviours, physiological changes in these regions will be examined to elucidate impacts on both the DA and circadian systems.

Chapter 1

The first experiment presented in this thesis utilized the above -mentioned KO of *Bmal1* in the LHb. Mice were given three weeks to recuperate and ensure ample time to viral injection before testing. Behavioural assessments focusing on affective and motor behaviours were conducted, specifically a battery of tests including the open-field test, sucrose preference tests, tail suspension test, horizontal bar test, pole test, and rotarod. After testing tissue samples from the dorsal striatum (DS) and SN were collected at four time-points throughout the day, ZT 2, 8, 14, and 20. Tissue samples were analyzed using real-time PCR, targeting clock and DAergic functioning. Additionally, liquid chromatography coupled with mass spectrometry was used to quantify DA levels in striatal tissue. The findings revealed significant motor deficits and disrupted rhythms in both the SN and DS.

Chapter 2

Building on these results, we aimed to rescue the observed motor deficits in LHb *Bmal1* knockout mice using both pharmacological and non-pharmacological interventions. The pharmacological approach involved daily injections of the D2 agonist quinpirole, while the non-pharmacological approach utilized running wheels. Motor performance was assessed before and after the interventions. Both methods moderately improved motor phenotypes but did not entirely restore molecular functioning in the SN and DS. These findings suggest that interventions targeting rhythmic functioning can alleviate LHb *Bmal1* induced motor deficits.

Chapter 3

To gain a broader understanding of the impact of our LHb *Bmal1* KO on DAergic functioning, our final project examined the mesolimbic reward pathway, specifically targeting alcohol drinking behaviour. We assessed LHb-specific *Bmal1* knockout mice using various alcohol drinking paradigms, including binge drinking, intermittent access, aversive drinking, and forced abstinence. The results revealed a sexually dimorphic impact of the *Bmal1* KO on drinking behaviour: male KO mice consumed more alcohol than controls, while female KO mice drank less.

Conclusions

In summary, this thesis investigates the interaction between DA regulation and the circadian system, focusing on their roles in motor functioning and addiction-like behaviours. By exploring the interactions between the circadian and DAergic systems, this research aims to deepen our understanding of the mechanisms underlying their shared behaviours. Ultimately, this could enhance our knowledge of how these systems interact in the context of abnormal behaviours and lead to improved therapeutic interventions.

Chapter 1

The lateral habenula affects motor function in mice through the regulation of daily rhythms in the nigrostriatal pathway

Cassandra Goldfarb, Nadav Baharav, Heng Jiang, Amanda Szubinski, Shimon Amir, Konrad Schöttner

Abstract

Alterations to the dopaminergic system can have several consequences on behaviour – such as the development of disorders like addiction, schizophrenia, and Parkinson's. The habenula (LHb) is uniquely positioned to act on a large source of dopaminergic (DA) neurons, sending inhibitory signals both directly and indirectly to the ventral tegmental area (VTA) and the substantia nigra (SN). Additionally, the LHb houses a circadian clock that appears to function independently from the central circadian pacemaker. We investigated the role of the LHb in acting as a pacemaker for the production and release of DAergic signals along the nigrostriatal pathway. Using male and female *Bmal1* floxed mice, we stereotaxically injected AAV-2/9 Cre-eGFP virus into the LHb to selectively knockout *Bmal1*. We found a significant impact on motor functioning in both male and female knockout mice. Daily rhythms of gene expression, primarily targeting the circadian clockwork and DA synthesis, and liquid chromatography coupled mass spectrometry for striatal DA measurements were utilized to assess molecular functioning. Results indicate blunting of rhythms in the dorsal striatum (DS) and (SN) of knockout animals, that may contribute to the observed behavioural phenotype. As proper functioning of the striatum is presumably maintained by a mutual interaction of the circadian clock and DAergic system, these findings support that disrupting the LHb clock can impact functioning along the nigrostriatal DA pathway.

Key words

Habenula, circadian rhythms, Bmal1, movement, DA, gene expression

Introduction

Animals exhibit daily rhythms in physiology and behaviour generated by an internal timekeeping system comprising an interconnected network of biological clocks in the brain body. The system provides a temporal order of biological processes according to the time of day, allowing organisms to anticipate challenges imposed by the cyclic changes in the environment (de Assis & Oster, 2021).

Tissue-specific clocks generate daily rhythms through the expression and reciprocal interaction of circadian clock genes and their protein products (King & Takahashi, 2000). Because these oscillations vary in phase, period, and stability, they must be constantly reset to remain aligned with rhythms of other internal processes and the external environment. Through mutual interactions of central and peripheral clocks within an organism, the circadian system provides the temporal integrity of metabolic, endocrine, and behavioural functions needed to ensure systemic homeostasis in a rapidly changing environment, which is critical health and well-being (Dibner et al., 2009).

It is proposed that diurnal changes in neuronal networks and signalling pathways within the brain, including the synthesis and release of neurotransmitters such as dopamine (DA), are crucial for the regulation of behavioural processes. Studies on the dorsal striatum (DS), the major input structure of the basal ganglia, revealed a clear link between circadian dysregulation and altered behavioural phenotypes in animal models (de Zavalia et al., 2021; Landgraf et al., 2016; Ozburn et al., 2017; Schoettner et al., 2022). This view has been supported by studies on human brain tissue from donors with diagnosed mental illness (Ketchesin et al., 2023). Despite the significance of diurnal rhythms in striatal function, however, relatively little is known about how these oscillations are sustained, given that nuclei of the basal ganglia are not intrinsically rhythmic (Landgraf et al., 2016) and lack direct projections from the SCN (Dibner et al., 2009; Iijima et al., 2002). It has therefore been proposed that temporal cues must be relayed through autonomous or semi-autonomous oscillators located in separate brain areas (Becker-Krail et al., 2022; Pradel et al., 2022).

Among several extra-SCN oscillators that have been identified in the brain, the lateral habenula (LHb) has emerged as a promising target involved in the regulation of daily rhythms in the basal ganglia. Neurons of the LHb display intrinsic daily rhythms in clock gene expression and electrophysiological activity through input from hypothalamic regions, including the SCN

and the retina (Salaberry et al., 2019; Zhang et al., 2016). Moreover, the LHb exerts direct and indirect control over the ventral tegmental area (VTA) and substantia nigra (SN), thus affecting the rhythms of DAergic circuits.

Recent studies have highlighted the role of DA in the regulation of diurnal rhythms in the basal ganglia (Brami-Cherrier et al., 2020; Hood et al., 2010; Imbesi et al., 2009; Pradel et al., 2022; Sahar et al., 2010; Uz et al., 2005). Levels of extracellular DA in the striatum vary across the 24-hour day (Ferris et al., 2014; Paulson & Robinson, 1995; Y. Smith & Kieval, 2000) as a result of daily changes in DA synthesis and release. The expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA production, varies across the day (Chung et al., 2014), just like levels of monoamine oxidase A (Maoa) and DA transporters (DAT) in the striatum (Ferris et al., 2014; Hampp et al., 2008). Notably, daily oscillations in DA levels affect rhythms in clock protein expression in medium spiny neurons (MSNs) of the DS (Hood et al., 2010), suggesting that diurnal changes in DA tone within the nigrostriatal pathway serve as a timing cue to synchronize and maintain striatal rhythms.

We therefore conceptualize a pacemaking function of the LHb that drives diurnal oscillations of DA levels in the nigrostriatal pathway, thereby affecting clock function in downstream brain regions, such as the DS. We tested this hypothesis by a series of experiments utilizing a mouse model with a conditional knockout of *Bmal1* within the LHb. Our work demonstrates that mice with a LHb specific deletion of *Bmal1* display blunted rhythms of striatal DA, which is likely caused by a dysregulation of daily rhythms of DA synthesis in the SN. Strikingly, these alterations are associated with attenuated motor functioning in male and female mice. Therefore, this work demonstrates the critical role of the LHb circadian clock in the coordination of DAergic functions in the basal ganglia and the regulation of behaviour.

Results

Bmal1 ablation from the LHb reduces wheel running activity but does not influence central clock function and affective behaviours

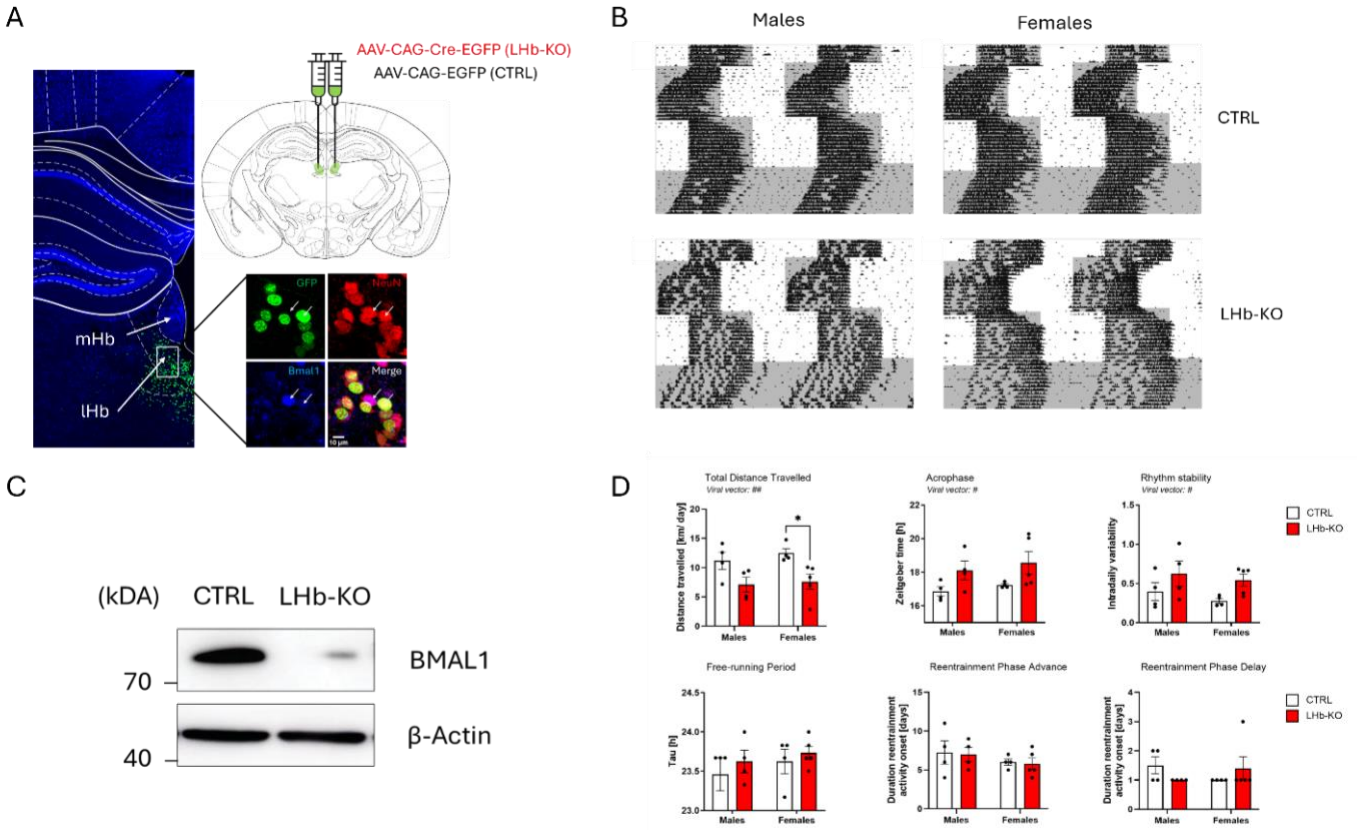

Figure 1-1. Bmal1 was successfully knocked out in the LHb. A) Representative immunofluorescence staining image of GFP expression in LHb of mice receiving stereotaxic injections of AAV-CAG-Cre-EGFP viral vector. 60x representative immunofluorescence staining in LHb tissue of a knockout mouse GFP (green), NeuN (red), and BMAL1 (blue) immunofluorescence staining in LHb tissue of a knockout mouse. Arrows demonstrate no overlap between GDP and BMAL1 in neurons. B) Representative actograms of mice under 12:12 LD after a 6-hour phase advance, 6-hour phase delay, and under constant darkness. C) Western blotting results levels of BMAL1 in pooled LHb tissue of KO and Ctrls, n = 4 per group. D) Running wheel data (males: n=8 (4KO); females: n=9 (5 KO)) was analyzed using Clocklab software, graph images depicting two-way ANOVA results for viral vector x sex: average daily distance travelled (*p< .05), acrophase, rhythm stability, free-running period, and time to entrain after a phase advance and phase delay are depicted.

Bilateral intracerebral infusion of Cre-expression viral vector led to a successful ablation of *Bmal1* from the LHb. Immunofluorescence imaging confirmed the absence of BMAL1 protein from GFP labelled cells of the LHb exclusively (Fig. 1A). Western blot analysis furthermore demonstrated the absence of BMAL1 from tissue of the LHb collected from animals infused with virus expressing Cre protein when compared to controls (Fig. 1C).

To verify that the deletion of *Bmal1* did not affect central clock function, animals were kept in cages equipped with running wheels to study circadian rhythms of locomotor activity

under various light/dark conditions. While free-running periods and re-entrainment durations following 6-h phase advances and 6-h phase delays of the light/dark cycle were unaffected by the deletion of *Bmal1* from the LHb (Fig. 1D, lower left, mid and right panels, Table. 1), levels of wheel running were reduced in knockout animals (Fig. 1D, upper left panel, Table. 1). Concomitant to low activity levels in KO mice was a reduced rhythm stability and a shift of the central phase of the 24-h activity cycle. Particularly, intradaily variability was increased and the acrophase was delayed in KO mice (Fig. 1D, upper mid and right panel, Table. 1), which was also demonstrated by activity patterns (Fig. 1B).

Affective behaviours assessed in the open field and sucrose preference test were unchanged in KO animals compared controls (Suppl. Fig. 1A-C), suggesting that the knockout of *Bmal1* from the LHb did not cause behavioural abnormalities in general. However, an effect of the viral vector on the immobility time in the tail suspension test was found ($F_{(1, 29)} = 5.029$, $P = 0.0327$, two-way ANOVA) indicating that KO mice move less when suspended by their tails (Suppl. Fig. 1D).

Table 1-1. Results of statistical analysis of wheel running activity and motor function in CTRL and KO mice

Locomotor activity rhythm	**Two-way ANOVA**		
	Sex	Viral vector	Interaction
Total distance travelled	n.s.	$F_{(1, 13)} = 12.36$, $p = 0.0038$	n.s.
Acrophase	n.s.	$F_{(1, 13)} = 6.795$, $p = 0.0217$	n.s.
Intradaily variability	n.s.	$F_{(1, 13)} = 5.613$, $p = 0.0340$	n.s.
Free-running period	n.s.	n.s.	n.s.
6-h phase advance	n.s.	n.s.	n.s.
6-h phase delay	n.s.	n.s.	n.s.
Motor test	Two-way ANOVA		
	Sex	Viral vector	Interaction
Horizontal bar test	n.s.	$F_{(1, 29)} = 70.96$, $p < 0.0001$	n.s.
Pole test	n.s.	$F_{(1, 29)} = 68.15$, $p < 0.0001$	n.s.
Rotarod test	n.s.	$F_{(1, 29)} = 22.78$, $p < 0.0001$	$F_{(1, 29)} = 5.889$, $p = 0.0217$

Deletion of *Bmal1* from the LHb attenuates motor function and blunts oscillations of striatal DA levels and clock gene expression

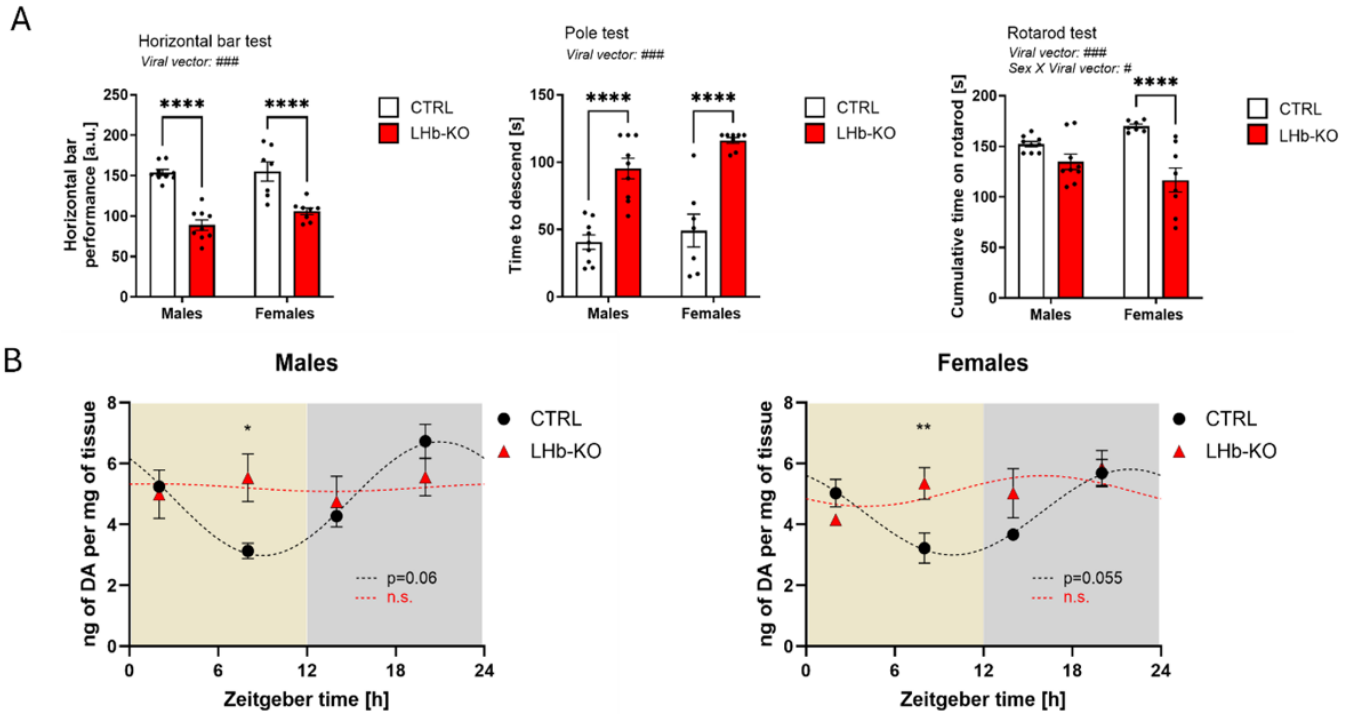

Figure 1-1-1. Striatal DA levels and motor functioning in LHb Bmal1 KO mice. *A) Two-way ANOVAs were run for the: horizontal bar test, pole test, and rotarod. Males: n = 18 (9 KO); females: n = 15 (8 KO). B) Two-way ANOVAs were run for liquid chromatography coupled mass spectrometry measurements of striatal dopamine levels. Males and females: n = 6 (3 KO).* Note: *p< .05; ** p<.01; ****p<.0001

Due to low wheel running activity observed in mice with habenular *Bmal1* ablation, motor functioning was furthermore evaluated using the horizontal bar test, pole test and rotarod test. All tests demonstrated a significant deficit in motor function in KO mice. Specifically, male and female KO mice performed worse in the horizontal bar and pole test compared to controls (Figs. 2A, left and mid panel, Table. 1). While the type of viral vector was decisive for rotarod performance, only KO females executed the test significantly worse compared to controls (Fig. 2A, right panel, Table. 1). Because motor functioning is modulated through the midbrain DAergic system, DA levels in tissue collected from the dorsal striatum at various times of the day were assessed using HPLC in a follow-up experiment.

Table 1-2. Results of statistical analysis of daily striatal dopamine fluctuations in CTRL and KO animals.

Sex	Viral vector	One-way ANOVA Time point	
Males	CTRL	$F_{(3, 8)} = 23$, $p = 0.0003$	
	KO	n.s.	
Females	CTRL	$F_{(3, 8)} = 7.86$, $p = 0.009$	
	KO	n.s.	
Sex	**Viral vector**	**Two-way ANOVA Time point**	**Interaction**
Males	n.s.	$F_{(3, 16)} = 3.914$, $p = 0.0285$	$F_{(3, 16)} = 3.395$, $p = 0.0438$
Females	n.s.	$F_{(3, 16)} = 3.910$, $p = 0.0286$	$F_{(3, 16)} = 3.527$, $p = 0.0392$

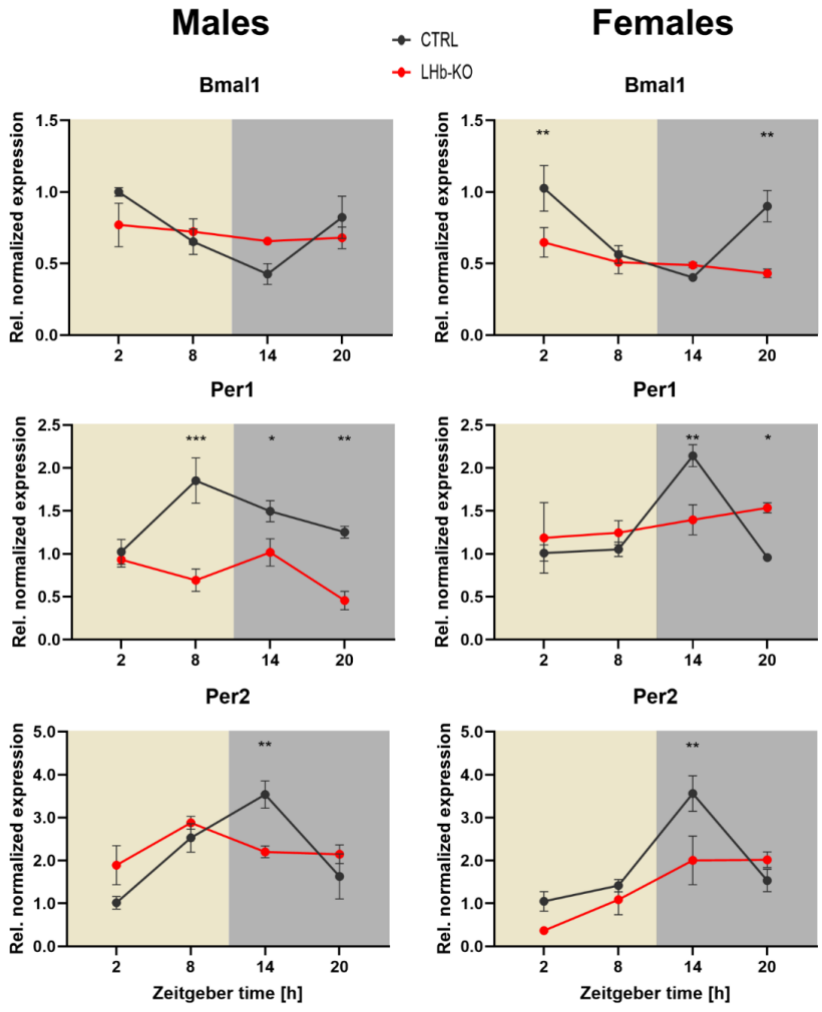

Figure 1-1-2. DS clock gene expression is blunted in KO mice. Two-way ANOVAs were run for all gene expression data, viral vector x time. Males: n = 6 (3 KO); females: n = 6 (3 KO). Top row: daily Bmal1 levels in males (left) and females (right). Middle row: Per1 rhythms in males (left) and females (right). Bottom row: Per2 rhythms males (left) and females (right). Note: *p< .05; ** p<.01; ***p<.001

Control mice displayed robust diurnal changes in DA levels peaking around the end of the dark phase (Fig. 2B). One-way ANOVA revealed a significant effect of daytime on DA levels in both CTRL males and females (Table. 2). Subsequent cosine analysis confirmed diurnal fluctuations of DA levels, although the results of zero-amplitude testing approached levels of statistical significance only (Table. 3).

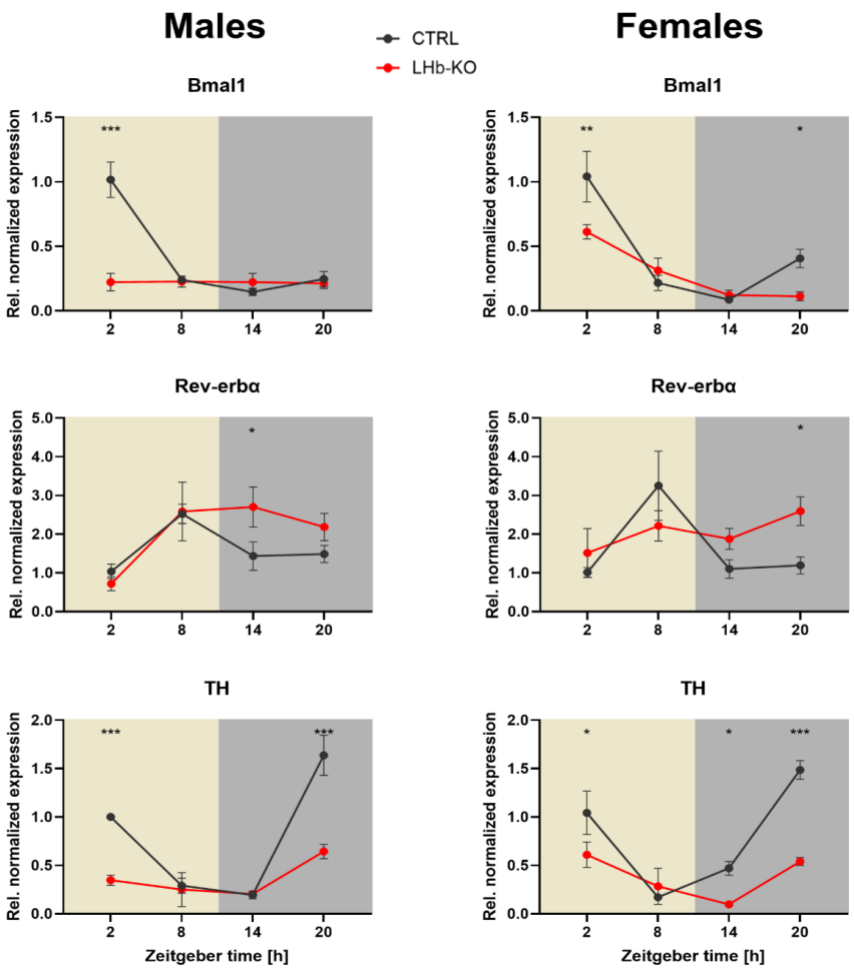

Figure 1-1-3. LHb Bmal1 KO altered SN gene expression. Two-way ANOVAs were run for all gene expression data, viral vector x time. Males: n = 6 (3 KO); females: n = 6 (3 KO). Top row: daily Bmal1 levels in males (left) and females (right). Middle row: Rev-erbα rhythms in males (left) and females (right). Bottom row: Tyrosine hydroxylase rhythms in males (left) and females (right). Note: *p< .05; ** p<.01; ***p<.001

In contrast, KO animals displayed considerably blunted oscillations of DA levels across the day (Fig. 2B), which was confirmed by statistical analysis (Table. 3). Two-way ANOVA (factors: time of day, viral vector) furthermore verified that in both males and females, time of day was significantly affecting levels of DA in the dorsal striatum (Table. 2). Importantly, a significant interaction between time of day and the type of viral vector was found (Table. 2), further indicating that diurnal fluctuations in striatal DA depend on the presence of *Bmal1* in the LHb.

Table 1-3. Cosine analysis of diurnal changes in striatal dopamine levels measured by HPLC

Sex	Viral vector	Mesor	Amplitude	Acrophase (ZT)	Zero Amplitude test		
					F	**P**	**R^2**
Males	CTRL	4.85	1.87	20.99	115.8	0.065	0.995
	KO	5.2	0.12	1.74	0.03	0.96	0.06
Females	CTRL	4.4	1.41	21.92	161.4	0.055	0.997
	KO	5.1	0.5	16	0.246	0.818	0.33

To decipher the consequences of disrupted striatal DA oscillations on MSN function, gene expression analysis was performed. Because previous work has established a link between DA signalling and circadian clock gene expression, daily profiles Bmal1, Per1 and Per2 were examined. Control males and females displayed robust diurnal fluctuations in striatal clock gene expression, whereas expression profiles were blunted in animals with a deletion of Bmal1 from cells of the LHb (Fig. 3). Statistical analysis by two-way ANOVA revealed a significant effect of daytime on expression profiles, although a significant interaction between time of day and viral vector indicated that oscillations of striatal clock gene expression were dependent on habenular Bmal1 expression (Table. 4).

Table 1-4. Results of statistical analysis of striatal gene expression profiles.

Gene	Sex	Two-way ANOVA		
		Viral vector	Time point	Interaction
Bmal1	Males	n.s.	$F_{(3, 16)} = 4.498$, $p = 0.018$	n.s.
	Females	$F_{(1, 16)} = 11.18$, $p = 0.0041$	$F_{(3, 16)} = 7.776$, $p = 0.002$	$F_{(3, 16)} = 4.646$, $p = 0.0161$
Per2	Males	n.s.	$F_{(3, 16)} = 9.035$, $p = 0.001$	$F_{(3, 16)} = 4.841$, $p = 0.0139$
	Females	$F_{(1, 16)} = 5.452$, $p = 0.0329$	$F_{(3, 16)} = 15.73$, $p < 0.0005$	$F_{(3, 16)} = 3.593$, $p = 0.0371$
Per1	Males	$F_{(1, 16)} = 37.24$, $p < 0.0005$	$F_{(3, 16)} = 4.016$, $p = 0.0262$	$F_{(3, 16)} = 4.844$, $p = 0.0139$
	Females	n.s.	$F_{(3, 16)} = 5.927$, $p = 0.0064$	$F_{(3, 16)} = 4.936$, $p = 0.0130$

Blunted striatal DA levels in mice with *Bmal1* deletion for the LHb are associated with changes in genes expression in the substantia nigra

Because extracellular DA levels in the dorsal striatum are regulated by DArgic neurons of the substantia nigra pars compacta, gene expression levels of circadian clock genes and tyrosine hydroxylase were assessed. While marked diurnal changes in of *Bmal1*, *Reverb-a* and *Th* expression were observed in both male and female control mice, levels of gene transcripts were blunted in KO mice (Fig. 4). Statistical analysis furthermore demonstrated that the presences of *Bmal1* in the LHb is a critical factor in the regulation of daily changes in *Bmal1* and *Th* expression in the substantia nigra (Table. 5). Although visual analysis of *Reverb-a* expression profiles indicated an impact of habenular *Bmal1* deletion, only time of day had a significant

effect on diurnal levels of *Reverb-a* transcripts (Table. 5).

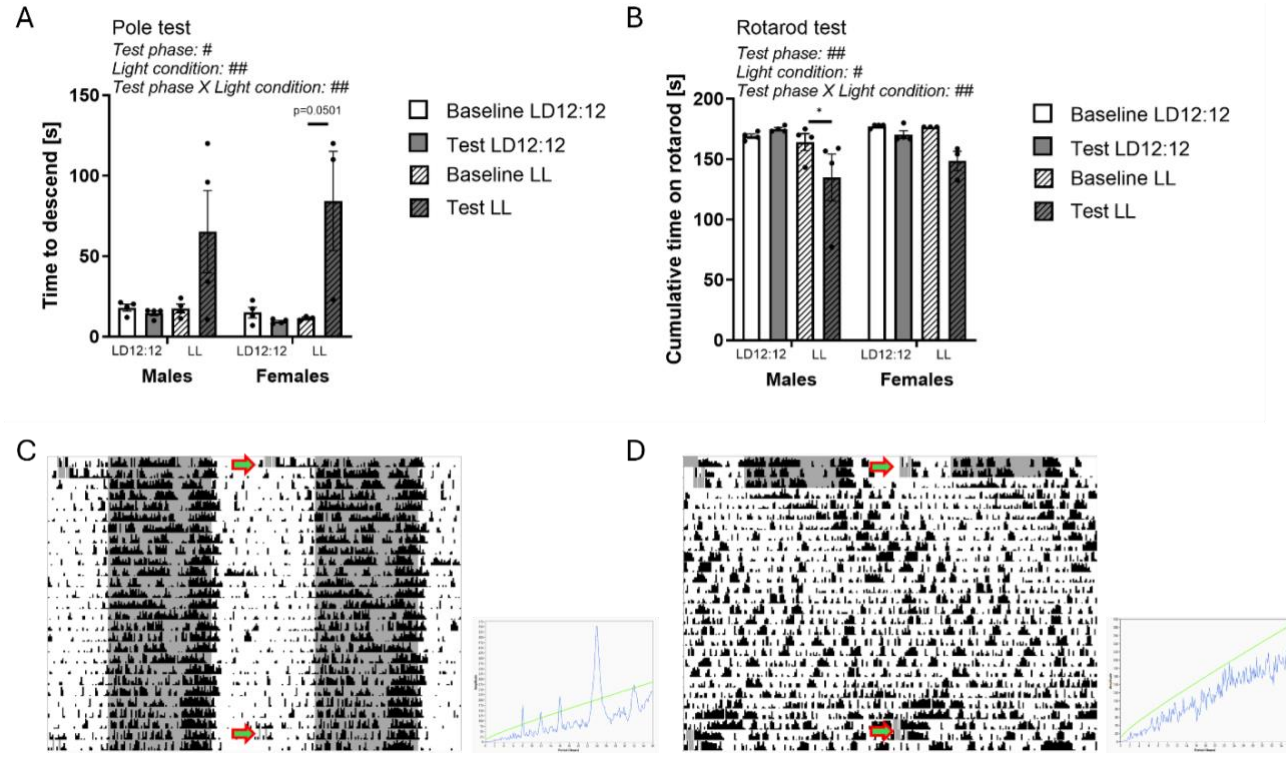

Figure 1-1-4. Mice under constant light performed worse on motor tests. Males: n = 8 (4 LL); females: n = 7 (3 LL). A) Two-way ANOVA of pole test performance before and after constant light exposure, light x time. B) Two-way ANOVA of rotarod performance at baseline and after constant light exposure, light x time. Males: *p< .05. C) Sample actogram of a wild-type mouse under typical 12:12 LD cycle. Arrows depict motor testing. D) Actogram of wild-type mouse kept under constant light for 3 weeks. Arrows show times of behavioural testing.

Global rhythm disruption causes motor phenotypes resembling effects of *Bmal1* deletion from the LHb

Table 1-5. Results of statistical analysis of substantia nigra gene expression profiles.

Gene	Sex	Two-way ANOVA		
		Viral vector	Time point	Interaction
Bmal1	Males	$F_{(1, 16)} = 16.3$, $p = 0.001$	$F_{(3, 16)} = 18.74$, $p \leq 0.0005$	$F_{(3, 16)} = 18.31$, $p \leq 0.0005$
	Females	$F_{(1, 16)} = 5.578$, $p = 0.0312$	$F_{(3, 16)} = 26$, $p \leq 0.0005$	$F_{(3, 16)} = 4.161$, $p = 0.0234$
Reverb-a	Males	n.s.	$F_{(3, 16)} = 6.207$, $p = 0.0053$	n.s.
	Females	n.s.	$F_{(3, 16)} = 3.973$, $p = 0.0272$	n.s.
Th	Males	$F_{(1, 16)} = 30.84$, $p \leq 0.0005$	$F_{(3, 16)} = 32.98$, $p \leq 0.0005$	$F_{(3, 16)} = 10.42$, $p \leq 0.0005$
	Females	$F_{(1, 16)} = 22.51$, $p \leq 0.0005$	$F_{(3, 16)} = 20.59$, $p \leq 0.0005$	$F_{(3, 16)} = 6.293$, $p = 0.005$

Although various studies demonstrated a link between altered DA signalling and deficits in motor functioning in mice, effects of chronodisruption on motor performance are currently unknown. Blunted oscillations of striatal DA levels in KO mice were accompanied by attenuated performance in motor tasks suggesting functional causality, which is further supported by results in mice with abolished 24-h rhythms. Mice kept under prolonged time in constant light lost 24-h rhythmicity (Fig. 5C & D) and performed worse in the rotarod and pole test compared to animals kept under a standard light/dark cycle (Fig. 5 A&B). Specifically, time to descend was increased in chronodisrupted males and females compared to controls (Fig. 5A), while performance on the rotarod was reduced (Fig. 5B). Three-way ANOVA revealed a significant effect of the light condition and test phase on the performance in both tasks, and a significant interaction between these two factors indicated that indeed the prolonged exposure to constant light was causal to the observed motor deficits (Table. 6).

Table 1-6. Statistical analysis of motor performance in chronodisrupted animals.

Motor test	Three-way ANOVA			
	Sex	Test phase	Light condition	Interaction
Pole test	n.s.	$F_{(1, 11)} = 9.278$, $p = 0.0111$	$F_{(1, 11)} = 10.59$, $p = 0.0077$	Test phase X Light condition: $F_{(1, 11)} = 12.52$, $p = 0.0046$
Rotarod test	n.s.	$F_{(1, 11)} = 14.48$, $p = 0.0029$	$F_{(1, 11)} = 5.18$, $p = 0.0439$	Test phase X Light condition: $F_{(1, 11)} = 13.27$, $p = 0.0039$

Discussion

Daily changes in brain and neuronal network functions are critical for the regulation of physiology and behaviour. Although various parts of the brain display diurnal rhythms, it has been shown that these oscillations dampen rapidly in the absence of rhythmic input (Begemann et al., 2020; Landgraf et al., 2016). Few brain regions besides the SCN, such as the lateral habenula (LHb), have autonomous or semi-autonomous clocks enabling persistent oscillation independently of temporal cues (Guilding & Piggins, 2007; Salaberry et al., 2019). Therefore, it has been proposed that the purpose of an extra-SCN oscillator in the LHb is to act as a circadian pacemaker for midbrain dopamine (DA) nuclei, thus driving daily oscillations in DAergic functions – which are pivotal for proper behavioural output, including mood, reward and motor functioning (Becker-Krail et al., 2022; Pradel et al., 2022; Proulx et al., 2014). The results of this

study support this view. The conditional knockout of *Bmal1* in the LHb dysregulated daily oscillations of *Bmal1*, *Reverb-a,* and *Th* expression in the substantia nigra (SN), which was associated with an absence of daily DA oscillations and rhythms of clock gene expression in the dorsal striatum (DS). These outcomes suggest that the LHb is the pacemaker for the nigrostriatal pathway, synchronizing the striatal clock through daily changes in tonic DA release from the SN, thereby regulating motor functioning in mice.

The significance of the relationship between the circadian system and midbrain DAergic circuits has been recognized in various studies (Becker-Krail et al., 2022; Pradel et al., 2022). Rhythm analysis in isolated midbrain DAergic nuclei, however, demonstrated that most of these nuclei have weak circadian clocks characterized by rapidly dampening oscillations in the absence of rhythmic input (Landgraf et al., 2016). Because the central pacemaker in the SCN does not innervate midbrain DAergic nuclei directly, it has been suggested that temporal cues must be relayed through extra-SCN oscillators in the brain (Guilding & Piggins, 2007), including from the LHb. Despite some discrepancy in the literature regarding its intrinsic rhythmicity (Guilding & Piggins, 2007; Landgraf et al., 2016; Salaberry et al., 2019), the LHb has been classified as a semi-autonomous oscillator receiving indirect SCN and retinal input (Baño-otálora & Piggins, 2017; Young et al., 2022). Notably, a link between LHb clock gene expression and its molecular and neurophysiological properties, especially in mood and reward-related processed has been established in the past (Li et al., 2021; Mendoza, 2017; Olejniczak et al., 2020; Sakhi, Belle, et al., 2014; Salaberry et al., 2019; Young et al., 2022). However, most of these studies have been conducted in global clock gene KO animals, which limits the conclusion that can be made about a direct effect of the LHb clock on the regulation of downstream brain regions. As such, the present study elected to use a targeted LHb- specific *Bmal1* KO to circumvent this issue. Because the deletion of *Bmal1* within the molecular clockwork confers to loss in rhythmicity of cellular functions including gene expression (Bunger et al., 2000), it can be concluded that the LHb clock in *Bmal1* KO mice is non-functional. Importantly, the results of running wheel locomotor activity in the present study demonstrate that the conditional *Bmal1* knockout did not affect central clock function. Central clock properties like free-running period and phase responses following advancing and delaying shifts of the light/dark cycle were unaffected by the KO. These outcomes therefore suggest that irregularities of diurnal rhythms in LHb efferent regions directly or indirectly, must stem from the LHb *Bmal1* knockout itself.

Despite the role of circadian clock genes *Per1* and *Per2* in the LHb in the regulation of affective behaviours (Li et al., 2021; Olejniczak et al., 2021; Young et al., 2022), only a weak association was found regarding the contributions of *Bmal1* in the present study. Mice with a conditional *Bmal1* knockout displayed slightly higher depressive-like behaviour in the tail suspension test compared to controls, whereas anhedonia-like behaviour was unchanged in the sucrose preference test. The overall lack of an aberrant affective phenotype follows similar literature where a conditional knockout of *Bmal1* in the forebrain did not affect anxiety-like behaviour in mice (Price et al., 2016), nor did targeted deletion of Bmal1 in the striatum impact performance on these same tests (Schoettner et al., 2022). Unlike deletions of *Bmal1*, clocks with single knockouts of *Per1* or *Per2* retain rhythmicity (Zheng et al., 2001). These outcomes therefore suggest that individual gene effects of *Per1* and *Per2* within the LHb account for changes in mood-related behaviours, conceivable through their impact on neuronal excitability (Young et al., 2022), while dysregulation of the habenular clock through *Bmal1* deletion has only minor impacts on mood and affect.

LHb *Bmal1* KO mice displayed strong deficits in motor functioning. Wheel running activity was markedly reduced in males and females after a deletion of *Bmal1* in the LHb and this was then supported by poor performance in the rotarod, horizontal bar, and pole tests. However, it is important to note that spontaneous activity assessed in the open field test was unaltered in KO animals, suggesting normal baseline motor functioning. The authors of a study on the genetic ablation of the dorsomedial habenula, which revealed almost identical motor phenotypes, argued that impairments in both, wheel running activity and rotarod performance are due to a lack of motivation rather than a motor deficit (Hsu et al., 2014). The results of the present study, however, contradict this view. Sucrose preference in *Bmal1* LHb KO mice was unchanged despite a prolonged fasting period prior to the test, indicating similar levels of motivation in KOs and controls. Moreover, the rotarod, pole and horizontal bar tests are measures of motor coordination and execution, and their potential association with motivated behaviours is inconsistent (Campos et al., 2013; Liebetanz et al., 2007). As such, it can be concluded that factors other than simply a lack of motivation must therefore contribute to the motor deficits found in *Bmal1* KO mice.

Consistent with the results of the present study, a reduction of wheel running activity has been observed in rats treated with 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle

(Hood et al., 2010). Similarly, there is clear evidence showing the direct effects of DA deficiency on rotarod and pole test (Glajch et al., 2012; Ma & Rong, 2022; N. Ogawa et al., 1985; Sedelis et al., 2001), indicating that altered levels of extracellular DA in the striatum may be causal to the observed motor phenotypes. Unlike KO animals, mice with unilateral 6-OHDA lesions or MPTP treatment, which results in a loss of striatal dopamine, typically display reduced spontaneous activity in the open field test (Fredriksson et al., 2011; Slézia et al., 2023). However, DA levels measured in the dorsal striatum in KO mice were not reduced per se, but their amplitude was markedly decreased, indicating that daily oscillations in DA tone were blunted. Indeed, numerous studies have supported the presence of daily DA oscillations in midbrain DAergic circuits, particularly in the striatum, and it has been suggested that a change in DA tone across the day has implications in the regulation of DAergic functions (Pradel et al., 2022). The outcomes of the motor tests conducted in the present study therefore indicate that a dysregulation of daily DA oscillations, rather than a loss of DA, may be sufficient to attenuate motor functioning in *Bmal1* KO mice. Indirect support for this hypothesis was provided by the outcomes of motor tests conducted in arrhythmic animals, showing that a systemic dysregulation of circadian rhythms can impact motor functioning.

Unknown to date is the underlying mechanism leading to motor dysfunction in KO mice. Rhythms of clock gene expression and DA in the DS were blunted in KO mice. Because DA signaling affects clock gene expression in the striatum (Hood et al., 2010; Imbesi et al., 2009), it is tempting to speculate that daily changes in DA tone could be a zeitgeber to the striatal clock, which rapidly dampens in the absence of external cues (Landgraf et al., 2016). This, in turn, would indicate that the loss of rhythmicity in striatal DA tone may be causal for blunted rhythms of clock gene expression in the striatum. Interestingly, results from previous studies suggest that circadian clock gene expression in the striatum contributes to the regulation of motor function in mice (Schoettner et al., 2022). Ablation of *Bmal1* from medium spiny neurons (MSNs) of the striatum was associated with strongly attenuated motor performance in the horizontal bar and rotarod test. This effect appeared to be limited to the deletion of *Bmal1*, as motor performance was unaffected in mice with a conditional knockout of *Per2* (Schoettner et al., 2022). However, while motor coordination was affected by the conditional *Bmal1* knockout, voluntary wheel running activity was unchanged and spontaneous activity assessed in the OFT was increased (de Zavalia et al., 2021a; Schoettner et al., 2022). The discrepancy in these results could be attributed

to the differences in the levels of striatal *Bmal1* expression. While mice with striatum-specific *Bmal1* KO have no functional copies of the gene in MSNs (Schoettner et al., 2022), *Bmal1* was still expressed in KO mice here. Although it has been shown that loss-of-function of *Bmal1* in the striatum affected behaviour differently compared to its downregulation (Porcu et al., 2020), future experiments have to clarify the significance of potential dose effects of *Bmal1* in MSN in the context of motor functioning.

Alternatively, alterations in motor functioning, such as wheel running activity in *Bmal1* KO mice, may stem from changes in signaling pathways other than midbrain DAergic systems. The LHb also innervates serotonergic (5-HT) nuclei such as the dorsal raphe nucleus (DRN), which in turn projects to nuclei in the basal ganglia including the SN and the striatum (Huang et al., 2019; Reed et al., 2013). While an association between 5-HT and motor functioning has been established in the past (Weber et al., 2009), follow-up studies must elaborate the link between the LHb, 5-HT, and motor functioning in more detail.

In summary, this work provides compelling evidence that the LHb is a circadian pacemaker within the nigrostriatal pathway. By coordinating rhythms of DA synthesis in the SN, it regulates daily oscillations of striatal DA tone responsible for synchronizing the striatal circadian clock, which is critical to proper motor functioning in mice. While other monoaminergic systems must be considered in the regulation of striatal functioning and motor control, this study provides a basis for future investigations in the underlying molecular and neurophysiological mechanism and brain circuits regulating motor function.

Method

Experimental model and subject details

Bmal1 floxed mice (B6.129S4(Cg)-Arntl^{tm1Weit}/J) were originally obtained from Jackson Laboratories. Male and Female mice were group-housed under a 12:12 hrs light-dark (LD) cycle at 21 ± 2°C and 60% relative humidity with food and water available *ad libitum* prior to the stereotactic delivery of viral vectors at 12 – 16 weeks of age. The bedding of the cages was changed every week. Animals were kept individually after the surgery under the same housing conditions as described above unless stated otherwise.

This study was conducted following the guidelines and requirements of the Canadian Council on

Animal Care (CCAC), approved by the Concordia University ethics committee (AREC number 3000256).

Stereotactic Surgery

Mice were anesthetized by intraperitoneal injection of Ketamine (100 mg/kg body weight) and Xylazine (10 mg/kg body weight) solution and received subcutaneous administration of Ketoprofen (5 mg/kg body weight) as post-operational analgesia. Animals were placed in a stereotaxic apparatus (KOPF, Tujunga, CA, USA) and received bilateral microinjections of recombinant viruses in the LHb (AP: -1.65, ML: 0.4, and DV: -3.1) using a 30-gauge needle attached to a 10 ul Hamilton syringe which was connected to a micropump (Pump 11 Pico Plus, Harvard Apparatus, Holliston, MA, USA). The injector was inserted at a 0° angle and the virus was delivered at a rate of 100 nl/min (150 nl total volume). For the generation of conditional knockout animals, recombinant viral vectors expressing Cre and eGFP (AAV2/9-CAG-Cre-eGFP, $1x10^{12}$ vg/ml) were injected. Control animals received viruses expressing eGFP only (AAV2/9-CAG-eGFP, $1x10^{12}$ vg/ml). The injector was left in place for five minutes following the injection to optimize diffusion. Mice were allowed to recover for three weeks before behavioural testing. LHb-specific expression of viral vectors was evaluated at the end of the experiment. Animals with missing, incomplete or off-region eGFP expression were excluded from all experiments.

Behavioural Testing

Open Field Test

The open field test was used to assess anxiety-like behaviour and motor activity. The test was conducted at Zeitgeber time 2 (ZT2, ZT0 represents the time of lights-on). After a 30-minute habituation period in the experimental room, mice were placed in the corner of the open field arena (45 x 45 x 60 cm, Panlab, Barcelona, Spain) facing the wall. The open field was equipped with infrared beams to track horizontal and vertical activity over 60 minutes using the ACTItrack software (Panlab, Barcelona, Spain). At the end of the session, animals were weighed and returned to home cages while the arena was wiped with 70% ethanol solution (v/v in tap water) to remove any residues and olfactory cues before the next set of animals were tested. Total

distance travelled, speed, resting time, rearing behaviour (vertical movements) as well as permanence time in the central and peripheral area of the open field and the latency to enter the central area was assessed.

Sucrose Preference Test

The sucrose preference test for assessing anhedonia in laboratory animals as a proxy of depressive-like behaviour. One week prior to the beginning of the experiment, animals were habituated to two drinking bottles containing tap water (50 ml Falcon tubes with sipper). To minimize the effects of neophobia as a confounding factor, animals were given two bottles of 1% sucrose solution (w/v in tap water) for 3 hours the day before testing, and the volume of consumed sucrose solution was assessed to ensure all animals were drinking. Food and water were removed from animal cages 14 hours before the sucrose test was conducted to increase the motivation to consume sucrose solution. Two hours after the beginning of the light phase on the test day (ZT2), food was returned and one bottle of water and one bottle of 1% sucrose solution were inserted into the cage. The bottles were left in the cage for one hour, at which time they were removed and weighed to determine the amount of sucrose consumed (g/kg body weight) and the preference for sucrose solution ($V_{\text{sucrose solution}}/(V_{\text{sucrose solution}} + V_{\text{water}})$).

Tail Suspension Test

In the tail suspension test, escape-related behaviour is quantified as a proxy for depressive-like phenotypes in mice. The apparatus consists of a metal stand on which mice were suspended by their tails while being videorecorded (Samsung Galaxy A5 2017) for 6 minutes. Animals were habituated to the conditions of the experimental room for 30 min before the beginning of the test, which was conducted at ZT8. To prevent mice from tail climbing during the test, climbstoppers were placed over the proximal part of the tail (Can et al., 2011). After completion of the 6 minutes trial period, animals were weighed and returned to their home cages. Two experienced researchers blind to the experimental conditions analyzed the video recordings using an on-screen stopwatch (Stopwatch+, Center for Behavioural Neuroscience, Atlanta, GA, USA). The total amount of mobility time (in seconds), defined as any movement of the body, was scored and subtracted from 360 seconds to determine immobility time.

Horizontal Bar Test

Motor coordination was assessed in a horizontal bar test. The experimental setup comprised a squared cardboard box (41 cm × 41 cm × 41 cm) with a metal bar (ø 2.5 or 5 mm) mounted on the top center of two opposite sides of the box. A cotton soft pad was placed on the bottom of the box to cushion animals falling from the bar. The test was conducted between ZT6 – 8 following a 30-minutes period to habituate animals to the experimental room. During the first stage of the test, mice were gently raised by their tail to the center of the 2.5 mm bar, which they had to grasp with the forepaws before being released. Within a 60 seconds trial period, mice had to either transverse the rod and touch one side of the box with a paw, stay on the rod for 60 seconds, or fall, while being video recorded (Samsung Galaxy A5 2017). The test was repeated three times with a 30 second interval between each trial, before it was conducted on the thicker rod (ø 5 mm) following the same experimental design. Videos were analyzed using a stopwatch and a score was calculated for each trial based on the mice's performance, i.e. the time to reach one of the criteria mentioned above. For transversing the bar, the score was calculated by subtracting the time reaching the end of the bar from 120 (score pass = 120 - time pass). The score for falling represented the same time it took the mice to fall from the bar (score fall = time fall). If a mouse stayed on the bar for the entire 60 second trial period, a score of 60 was given. A cumulative score from the averages of the three trials of each experimental stage was calculated.

Pole Test

The pole test is common test to assess motor function in mice. Animals were habituated to the experimental room for 30 minutes prior to the test, which has been conducted between ZT8-ZT10. Mice were placed head-upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm) placed in an enclosure filled with bedding (5 cm) to cushion animals falling from the pole. Mice were trained on how to descend the pole before testing began. Mice were given 120 seconds to descend the pole, time was stopped upon reaching the enclosure surface. Scores were averaged over three trails, with 30 seconds intertrial intervals. If mice failed to turn downward and descend the pole or if the mouse fell, time was taken as 120 second (default value). The criteria assessed includes the proportion of those who were able to complete the turn and the total time to descend to the ground over three trials. Animals were video recorded (Samsung Galaxy A5 2017) and the videos were scored by an experienced researcher blind to the experimental conditions using a stopwatch.

Rotarod Test

Motor coordination was furthermore assessed on a custom-made, fixed-speed rotarod (Concordia University, Montreal, Canada). The rotarod was composed of a rod (ø 5 cm) mounted 19.5 cm above the base of the device, which was separated into 6 individual compartments equipped with soft foam pads to cushion mice falling off the rod. Two flanges (ø 20 cm) separated a 9.3 cm interspace on the rod, which was driven by an electric motor. A gearbox was used to set the desired turning speed of the rod. On the test day, animals were habituated to the experimental room 30 minutes before they underwent a training session on the rotarod between ZT2–4 to get familiar with the apparatus. Animals were gently lifted by their tail and carefully placed on the rod turning at 2 rpm. Animals falling from the rod were put back on the rod immediately throughout the 5-minute training session. Animals returned to their home cages until the actual test was conducted between ZT6-8 at the same day. Mice were placed on the turning rod as described above and left on the rod for a 1-minute trial while being videorecorded. In contrast to the training session, mice were not placed back on the rod within the trial if they fell off the rod unless it was due to poor placement by the experimenter. In that case the trial was not counted and repeated after a short resting time. The trial was repeated two more times with 30 second intertrial periods before the rod was set to a higher speed. The starting speed of 2 rounds per minute (rpm) was gradually increased to 4, 8 and 12 rpm. Animals that failed to stay on the rod for 1 minute in all three trials were not tested further at higher speeds. Rotarod performance was given as a cumulative score calculated from the average times of the 3 trials at each speed.

Locomotor Activity Rhythms

To verify that the circadian system and gross locomotor activity was not affected by the deletion of *Bmal1* from the LHb, 15 weeks old mice were housed individually in cages equipped with running wheels (Actimetrics, Wilmette, IL, USA) food and water were provided *ad libitum*. Cages were placed in light-proof cabinets equipped with programmable lights (Actimetrics, Wilmette, IL, USA). At the beginning of the experiment, mice were kept under a standard 12:12 h light-dark (LD) cycle with the light intensity set to 200 lux during the light phase and 0 lux during the dark phase to assess basic measures of daily rhythms in locomotor activity. Thereafter, mice were exposed to 6 h phase advance (+6h), followed by a 6 h phase delay (-6h)

to evaluate the capacity of the circadian clock to adjust to phase changes of the LD cycle. Intrinsic properties of the circadian clock were assessed in animals exposed to constant darkness (DD). Mice were kept for 21-40 days under each respective light regimen. Wheel running was recorded and analyzed using Clocklab 6 (Actimetrics, Wilmette, IL, USA). Basic measures such as mesor, amplitude, activity onset and offset, and rhythm stability was analyzed from summed activity counts (10-minute bins) recorded over a period of 10 days. The time to re-entrain to 6-h phase shifts was determined for activity onset and offset individually by calculating the number of days from the shift of the light cycle until animals were stably alignment to the shifted light cycle. The free-running period was determined using Chi-Square periodogram in Clocklab 6 and double-plotted actograms were prepared to visualize representative locomotor activity patterns. Effects of central clock disruption on motor functioning was studied in a subset of male and female mice exposed to constant light. Animals were kept individually under standard housing conditions to assess motor function (rotarod and pole test) before they were randomly assigned into two groups. While one group of animals continued to be kept under standard conditions, the second group was exposed to constant light (~ 500 lx) for 3 weeks. Locomotor activity rhythms were assessed using infrared motion detectors mounted on top of the cages (Actimetrics, Wilmette, IL, USA) to determine the degree of chronodisruption of each animal visually and using the Chi^2-peiodogram in Clocklab 6. For this, the last 10 days before motor testing were analyzed. Animals of all groups were re-tested on the pole and rotarod test once mice kept under LL had no detectable rhythms anymore.

Tissue collection

Fresh frozen tissue was used for gene expression and LC-MS analysis. For this, brains from animals live-decapitated at corresponding times of the day were rapidly extracted and submerged in Isopentane cooled down to -30 °C for 3 minutes and stored at -80 °C thereafter. Serial coronal sections (100 µm thick) were obtained using a Cryostat (Microm HM 505 E, Microm International, Walldorf, Germany). The dorsal striatum (DS), the substantia nigra (SN) and the LHb were identified using the mouse brain atlas (Paxinos & Franklin, 2003), and tissue punches of the DS (1.5 mm in diameter) and SN (1 mm in diameter) were collected from each hemisphere. Region-specific expression of recombinant viral vectors was evaluated in brain sections of the LHb. The samples were kept in -80 °C until further processing. Formaldehyde-

fixed tissue was prepared for immunostainings. Animals were deeply anesthetized by exposure to an atmosphere of isoflurane and then transcardially perfused by cold saline (0.9% sodium chloride, pH 7.2) followed by paraformaldehyde solution (PFA, 4% in 0.1M phosphate buffer, pH 7.2) using an infusion pump. Brains were dissected and postfixed in PFA solution for 22 – 24h at 4°C thereafter. Coronal sections of brain tissue (30 um) were collected using a Leica vibratome (Leica Biosystems Inc., Concord, Ontario, Canada) and analyzed under a fluorescent microscope (Leica DM4000B, Leica Microsystems, Concord, Ontario, Canada) to validate region-specific viral vector delivery. Brain slices used for immunofluorescence imaging were stored at -20°C in Watson's cryoprotectant (Watson et al., 1986).

Liquid Chromatography Mass Spectrometry

2-(3,4-Dihydroxyphenyl)ethyl-1,1,2,2-d4-amine HCl (D4-DA, C/D/N ISOTOPES) was used as an internal standard in the extraction solution. The DA (DA) standards were prepared using pooled caudate putamen tissues (four wildtype mice, half female, half collected at ZT8 half at ZT20) to final concentrations of 0.05, 0.1, 0.5 and 1 μM of labelled DA standard. The calibration curve was plotted using the ratios of peak areas of DA (DA isotope) to D4-DA (labelled isotope) and the slope was used to calculate DA concentrations in samples. Briefly, tissue extraction was done using frozen brain tissue samples were thawed on ice and homogenized in 150 ul of an 80% methanol and isotope-labelled DA (to a final concentration of 2uM) in ultrapure water (v/v). The mixture was centrifuged at 4 °C, 14,000 rpm, for 20 min. The supernatant was transferred into a new tube and dried under nitrogen stream on ice. The residue was reconstituted in 1ml of 0.1M 3NT standard in ultrapure water (v/v).

Liquid chromatography mass spectrometry (LC-MS) analysis

LC-MS analyses were performed on an Agilent 1100 LC system coupled to a Thermo LTQ Orbitrap Velos mass spectrometer (Thermo Scientific™, Waltham, MA, USA) equipped with a heated electrospray ion source at positive mode. A Waters Atlantis dC18 column (100 x 2.1 mm, 3 μ particle diameter, Waters) was used and target compounds were eluted using an 18-min gradient at a flow rate of 250 μL/min with mobile phase A (99.9 % ultrapure water and 0.1 % formic acid (FA)) and B (99.9 % Acetonitrile and 0.1 % FA). The gradient started at 2 % B and held for 4 min, linear gradients were achieved to 90 % B at 6 min, then followed by isocratic

with 90 % B for 2 min and with 2 % B for 9 min. 5 µL of each sample was injected for the LC-MS analysis. MS spectra (*m/z* 100-250) were acquired in the Orbitrap at a resolution of 60000, DA and D4-DA were targeted at *m/z* 154.0863 and 158.1114 at retention time of 2.5 min. Peak area values were extracted using Thermo XCalibur software (v2.2 SP1.48).

Gene expression analysis

Total RNA was isolated from brain tissue of the DS and SN using a standard Trizol protocol according to the manufacturer's instructions (Ambion, Carlsbad, CA) and RNA yield was measured using spectrophotometry (Nanodrop 2000; Thermo Scientific™, Wilmington, DE, USA). Integrity of the isolated RNA was assessed by the "bleach gel" method (Aranda et al., 2012) and cDNA was synthesized from 1 ug of RNA using the iScript™ Reverse Transcription Supermix (Biorad, Hercules, CA, USA) thereafter. A no reverse transcriptase (no-RT) control was prepared along with the cDNA samples. Levels of gene expression were measured using SYBR green based quantitative real-time PCR. For this, 10 ul reactions containing SsoAdvanced Universal SYBR® Green Supermix (Biorad, Hercules, CA, USA) and 300 uM of the respective forward and reverse primers were amplified in triplicate using a CFX96™ Real-Time PCR system (Biorad, Hercules, CA, USA). The list of primers is provided in Table 1. The CFX Maestro Software (Biorad, Hercules, CA, USA) and Microsoft Excel were used to calculate relative normalized changes in gene expression by the delta-delta Ct (ΔΔCt) method (Pfaffl, 2001). The levels of target gene expression relative to two reference genes were further normalized to the sample collected from CTRL animals at ZT2.

Immunofluorescence staining

Free-floating sections kept in Watson's cryoprotectant were rinsed for 10 minutes in phosphate buffered saline (PBS, pH 7.4) followed by three rinses of 10 minutes each in a solution of PBS containing 0.3% Triton-X (PBST). The brain sections were kept in a blocking solution (3% skim milk powder, 6% normal donkey serum in PBST) for one hour and incubated in a PBST solution containing 3% skim milk powder, 2% normal donkey serum, rabbit Anti-BMAL1 primary antibody (1:500, NB100-2288, Novus Biologicals) and mouse Anti-NeuN primary antibody (1:2000, ab104224, Abcam) for one hour at room temperature thereafter. Sections were rinsed three times in PBST subsequently and incubated in a PBST solution

containing 3% skim milk powder, 2% normal donkey serum, donkey anti-rabbit IgG Alexa Fluor 647 secondary antibody (1:500, Thermo Scientific™) and donkey anti-Mouse IgG Alexa Fluor 594 secondary antibody (1:500, Thermo Scientific™) for one hour. Sections were rinsed three times in PBST and one time in PBS before they were mounted on microscope slides, cover slipped with a mounting media containing DAPI (ProLong™ Diamond Antifade Mountant, Thermo Scientific™) and stored at 4°C until imaging under a FluoView FV10i confocal laser scanning microscope (Evident Corporation, Tokyo, Japan). Subjects with missing, incomplete or off-region GFP signaling were excluded from the analyses.

Western Blot

Brain punches were lysed in NP40 lysis buffer containing 1% Nonidet P40, 0.1% SDS, 50 mM Tris base, 0.1 mM EDTA, 0.1 mM EGTA, and 0.1% deoxycholic acid, pH 7.4. A cocktail of inhibitors composed of 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 20 mM NaF, a protease inhibitor, and a phosphatase inhibitor (Sigma-Aldrich, P5726-1ML) at 1 mM was added to the NP40 buffer. The lysate was incubated under rotation for 30 minutes at 4°C. Proteins were found in the supernatant obtained after centrifugation at 14,000 rpm for 10 minutes at 4°C. Protein quantification was performed using the Pierce BCA Protein Assay Kit (ThermoScientific, #23227) with a BSA standard curve from 0 to 1 µg/µL to obtain a protein concentration of 30 µg. Proteins were mixed with 4X Laemmli loading buffer (Bio-Rad, #1610747) in a total volume of 60 µL and heated to 95°C for 5 minutes to denature the proteins. Protein samples were separated by SDS-PAGE on a 10% acrylamide gel using the Mini-PROTEAN Tetra System (Bio-Rad). Migration was performed in 1X Tris-Glycine electrophoresis buffer for 30 minutes at 100 V to gather the proteins, then one hour at 120 V for migration in the separation gel. The transfer was done onto a 0.2 µm nitrocellulose membrane (Bio-Rad, #1620112) for one hour at 100V in cold 1X Tris-Glycine-methanol transfer buffer. Membranes were blocked for 1 hour at room temperature in 5% TBST-BSA according to the manufacturer's recommendations. Blocked membranes were then incubated at 4°C with the primary antibody (2.5% TBST-BSA) until the next day before being incubated with the secondary antibody (1/3000) of rabbit or mouse (Goat Anti-Rabbit IgG (H+L) HRP Conjugate, Millipore, LV1646281, Goat Anti-Mouse IgG (H+L) HRP Conjugate, Millipore, AP124P) for one hour at room temperature. Proteins were detected using the Clarity Western ECL Substrate

detection system (Bio-Rad, #170-5061). Primary antibodies used are: Bmal1 (Novus Biologicals, NB100-2288, Dilution 1:1000, Rabbit) and b-actine (Sigma, A5316, Dilution 1 : 2000, Mouse). Densitometric analyses of immunoblots were performed using ImageJ software (Fiji, Version 2.1.0/1.53c).

Data analysis and Statistics

Prism 10 (Software, San Diego, CA, USA) was used to statistically analyze and visualize the results of this study. The outcomes were depicted as mean ± standard error of the mean (SEM). Results of behavioural tests were compared by two-way ANOVA (factors: sex and viral vector) followed by Sidak's multiple comparison. Outcomes of experiments conducted in constant light were compared using a three-way ANOVA (factors: sex, light condition and test phase) and analysed using Sidak's multiple comparison thereafter. To determine whether time of day affected diurnal levels of striatal DA, a one-way ANOVA was performed. Subsequently, a cosine analysis and zero-amplitude test were conducted to further assess the significance of daily oscillations in DA levels (Cornelissen, 2014). Daily profiles of gene expression and DA levels were analyzed using a two-way ANOVA (factors: viral vector and time point) followed by Tukey's multiple comparisons if data were normally distributed. The level of significance was set at $p = 0.05$.

Author contributions

K.S., S.A., and C.G. conceived and designed the study; C.G., N.B., A.S., and H.J. performed the experiments; C.G., K.S., and S.A. analyzed and interpreted the data, and wrote the manuscript; S.A. and K.S. Supervision.

Competing interests

The authors declare no competing interests.

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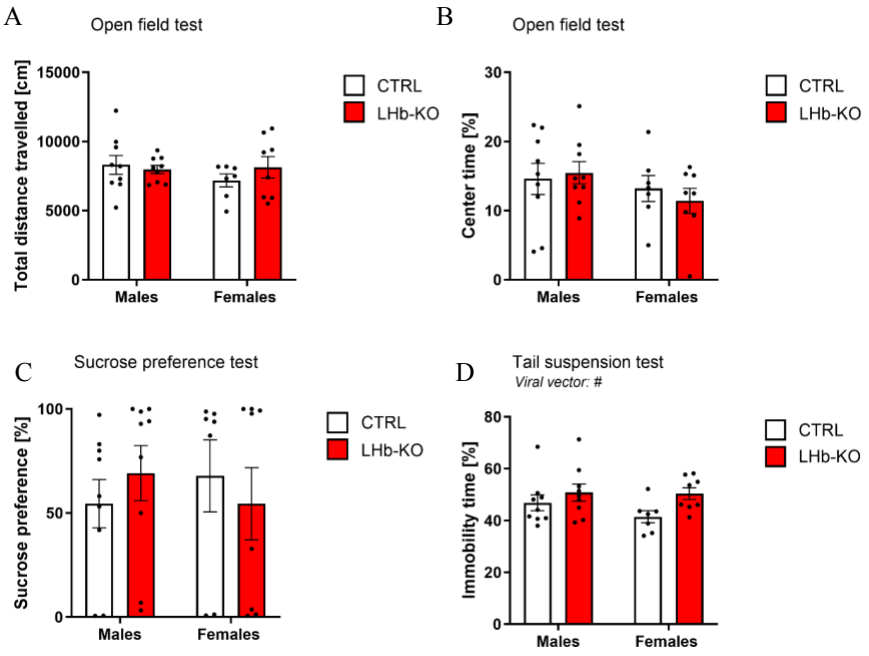
Supplemental information



Supplemental Figure 1. LHb Bmal1 KO does not meaningfully impact affective behaviours. Males: n = 18 (9 KO); females: n = 15 (7 KO). Two-way ANOVAs for Viral vector x sex was run for all tests. A) Open field test, distance travelled. B) Open field tests, time spent in center of the field. C) Sucrose preference test for a 1% sucrose solution. D) Time immobile during the tail suspension test.

Chapter 2

Evaluating Treatment Interventions on Motor Deficient Habenula *Bmal1* Knockout Mice

Cassandra Goldfarb, Nadav Baharav, Heng Jiang, Shimon Amir, Konrad Schöttner

Abstract

Circadian clocks play a pivotal role in coordinating cellular functions, such as metabolism and cell signaling. Previous studies have highlighted the significance of the nigrostriatal dopamine (DA) pathway in regulating the circadian functioning within the striatum (DS). Given the DS's hub-like role in the brain, striatal deviations have been linked to various disorders and diseases, including addiction, schizophrenia, and Parkinson's disease.

Previous research has identified the lateral habenula (LHb) as a pacemaker for daily rhythms of DA synthesis and release in the nigrostriatal pathway. The LHb, an SCN-independent oscillator projecting to both the ventral tegmental area (VTA) and substantia nigra (SN) and houses a semi-autonomous oscillator. We virally knocked out *Bmal1* specifically in the LHb of male and female *Bmal1* floxed mice. This revealed a substantial impact on motor coordination in both male and female knockout mice, alongside alterations in SN and DS functioning.

Presently, we assessed if daily timed injections of a D2 agonist, quinpirole, or voluntary running wheel activity could rescue this motor phenotype. Results of the quinpirole study indicated improved performance on the pole test in both males and females, with female- only rotarod improvement. Running wheel activity improved motor performance in both tasks and influenced DA levels in the striatum.

These findings suggest that both interventions exert differential impacts on motor functioning. Furthermore, they underscore the pivotal role of circadian rhythms in regulating motor function.

Keywords: Habenula, circadian rhythms, Bmal1, motor, running wheels, quinpirole

Introduction

Dopamine (DA), an essential neurotransmitter, plays a pivotal role in regulating behavioural processes, including reward, motor functions, and affect (Haber, 2014). Notably, due to clock controlled synthesizing enzymes in the substantia nigra (SN), DA shows rhythmic oscillations in regions such as the striatum (Ferris et al., 2014; Paulson & Robinson, 1995; Smith & Kieval, 2000).

These oscillations appear to be essential for striatal clock gene rhythms (Brami-Cherrier et al., 2020; Hood et al., 2010; Imbesi et al., 2009; Sahar et al., 2010; Uz et al., 2005). This effect was demonstrated previously when the loss of DA signals after 6-OHDA lesions to the medium forebrain bundle resulted in motor deficits and blunted *Per2* expression in the striatum (Hood et al., 2010). The critical role for DA in setting the striatal clock was further supported when daily timed injections of D2 receptor agonist, quinpirole, restored striatal rhythms and improved motor functioning (Hood et al., 2010). Interestingly, when clock functioning was abolished in the striatum after a striatal- specific *Bmal1* KO, mice also displayed significant motor impairments (Schoettner et al., 2022). These findings suggest that while DA is linked to motor functioning, this effect may be mediated through its interaction with the striatal clock.

This therefore highlights the importance of DAergic rhythms in the brain. Previously, we aimed to answer the question: what is acting as the pacemaker for DA rhythms? We investigated the clock in the lateral habenula (LHb), an epithalamic region implicated in the control of midbrain DA neurons (Ji & Shepard, 2007; Matsumoto & Hikosaka, 2007), and one of the few neural structures housing a semi-autonomous circadian clock (Salaberry et al., 2019). Using a targeted deletion of *Bmal1* in the LHb of mice, we demonstrated alterations in daily gene expression along the nigrostriatal pathway, blunted striatal DA levels, and significant motor deficits. These results suggest that the LHb clock plays a role in regulating DA rhythms and associated behaviours.

As such, the purpose of the present study was to rescue motor functioning in these knockout (KO) mice. The first intervention assessed the impact of four weeks of free access to running wheels, chosen due to their well-established feedback on the circadian system and the ability to synchronize rhythms (Bilu et al., 2022; Edgar et al., 1991; Edgar & Dement, 1991). The second intervention employed a pharmacological approach, utilizing daily timed injections of D2 receptor agonist, quinpirole, a previously successful technique in restoring striatal rhythms

after DA depletion (Hood et al., 2010). Male and female mice were assessed for motor performance at baseline and after interventions. The results indicate moderate restoration in motor functioning, SN gene expression, and levels of DA.

Results

Viral validation of Bmal1 knockout

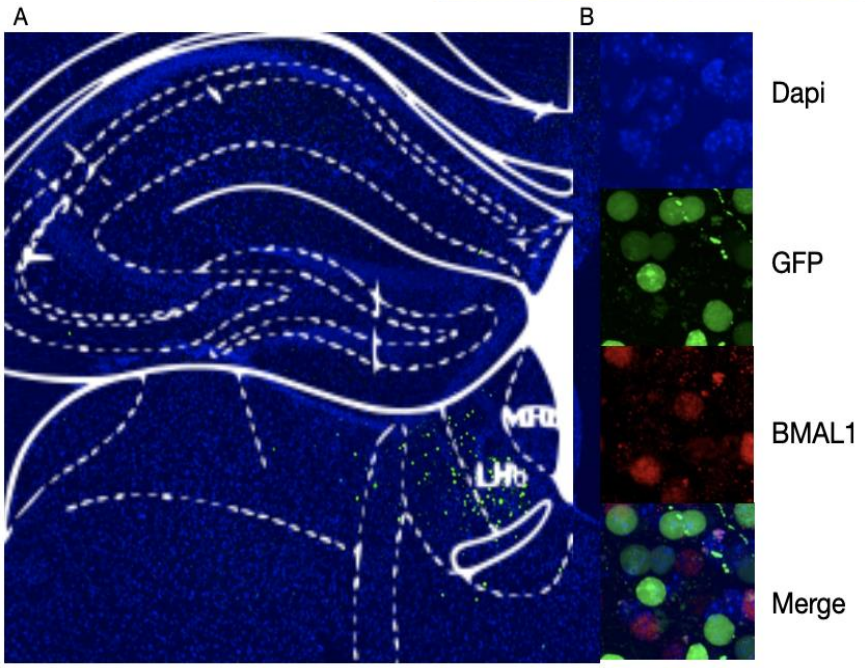


Figure 2-2-1. Immunofluorescence staining of a KO LHb mouse. A) Representative immunofluorescence staining imaged on a confocal microscope of GFP expression in LHb of mice receiving stereotaxic injections of AAV-CAG-Cre-EGFP viral vector. GFP staining is centralized in the LHb. B) 60x representative image of Dapi (blue), GFP (green), and BMAL1 (red) immunofluorescence staining in LHb tissue of a KO mouse.

Bmal1 floxed male and female mice received bilateral intra-LHb infusions of adeno associated virus (AAV) expressing Cre-recombinase and eGFP (KO) or AAV expressing eGFP only (Ctrl). Analysis of GFP expressing cells from histologically validated surgeries revealed that ~70% of cells in the LHb of experimental animals expressed Cre/eGFP (Fig. 1A). Immunofluorescence images confirm BMAL1 deletion in the habenula of KO mice (Fig. 1, B). Mice with low numbers of infected cell or off target infection were excluded from analyses.

Both interventions improved pole test performance

Baseline motor assessments began three-weeks after surgery, evaluating performance on the pole and rotarod tests. Interventions were run for a total of 4 weeks (an additional test at 2 weeks was included for the pharmacological intervention group). Both interventions resulted in significantly improved performance on the pole test in male and female KO mice (Fig. 2 A, B, E,

& F, Table 1).

Male mice with access to running wheels demonstrated significantly improved performance on the rotarod (Fig. 2C, Table 1). In females, all KO mice performed better at the test versus baseline on the rotarod, regardless of intervention (Fig. 2D, Table 1). However, running wheel usage improved rotarod performance to a greater extent than in those in standard home cages. This resulted in rotarod performance that was similar to control females (Fig. 2D, Table 1).

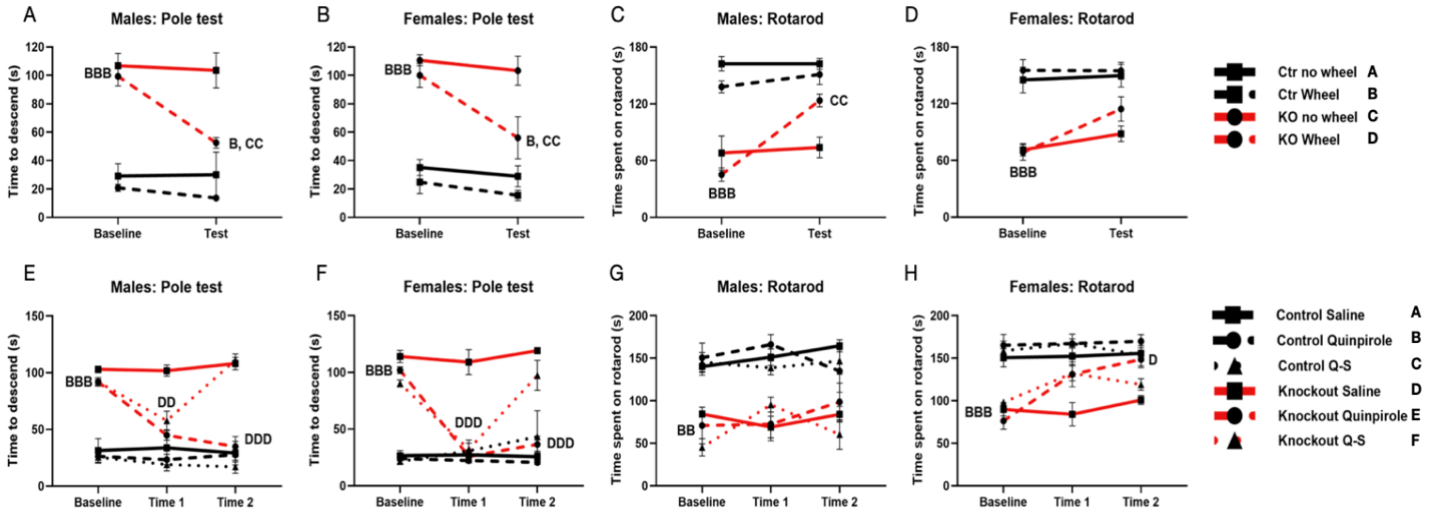


Figure 2-2-2. Motor performance in animals undergoing non-pharmacological or pharmacological interventions. *Non-*pharmacological (running wheels) Males: n= 24 (12 KO); females: n= 24 (12 KO). Pharmacological (Quinpirole) Males: n= 24 (12 KO); females: n= 24 (12 KO). 3-way ANOVAs were run for all tests. Analyses were separated by sex. (A-B) Nonpharmacological group pole test, time to descend; males (A) and females (B). (C-D) Nonpharmacological group rotarod, cumulative time spent on rod; males (C) and females (D). (E-F) Pharmacological pole test, time to descend; males (E) and females (F). (G-H) Pharmacological rotarod, cumulative time spent on rod; males (G) and females (H).
Notes. B= p<.05, BB= p<.01, BBB= p<.001

While both males and females improved on pole test performance after quinpirole injections, only female mice showed a significant improvement in rotarod performance (Fig. 2H, Table 1). Conversely, there was no significant improvement in males receiving quinpirole injections on rotarod performance (Fig. 2G, Table 1). Thus, improvement in the pharmacological intervention group was sex- and test- dependent.

Table 2-1. Behavioural results: Nonpharmacological group.

Test name			
Nonpharmacological		Male	Female
Pole test	Genotype	$F_{(1, 20)} = 69.92$, p=.001	$F_{(1, 20)} = 76.62$, p=.001
	Time	$F_{(1, 20)} = 14.94$, p=.001	$F_{(1, 20)} = 19.61$, p=.001
	Intervention	$F_{(1, 20)} = 6.702$, p=.018	$F_{(1, 20)} = 7.24$, p=.014
	Genotype x Time	$F_{(3, 16)} = 18.31$, p=.001	$F_{(3, 16)} = 4.161$, p=.023
	Time x Intervention	$F_{(1, 20)} = 12.55$, p=.002	$F_{(1, 20)} = 6.906$, p=.016
	Genotype x Time x Intervention	$F_{(1, 20)} = 5.889$, p=.025	$F_{(1, 20)} = 4.926$, p=.038
Rotarod	Genotype	$F_{(1, 20)} = 88.62$, p=.001	$F_{(1, 20)} = 15.28$, p=.001
	Time	$F_{(1, 20)} = 18.22$, p=.001	$F_{(1, 20)} = 18.69$, p=.001
	Intervention	$F_{(1, 20)} = 0.07$, p=.790	$F_{(1, 20)} = 1.04$, p=.321
	Genotype x Time	$F_{(3, 16)} = 9.76$, p=.005	$F_{(3, 16)} = 11.85$, p=.003
	Time x Intervention	$F_{(1, 20)} = 14.06$, p=.001	$F_{(1, 20)} = 1.95$, p=.178
	Genotype x Time x Intervention	$F_{(1, 20)}) = 6.764$, p=.017	$F_{(1, 20)} = 3.94$, p=.061
Pharmacological		Male	Female
Pole test	Genotype	$F_{(1, 18)} = 142.81$,p=.001	$F_{(1, 18)} = 97.19$, p=.001
	Time	$F_{(2, 36)} = 22.43$, p=.001	$F_{(2, 36)} = 4.02$, p=.027
	Intervention	$F_{(2, 18)} = 10.76$, p=.001	$F_{(2,18)} = 13.88$, p=.001
	Genotype x Time	$F_{(2, 36)} = 17.01$, p=.001	$F_{(2, 36)} = 25.02$,p=.001
	Time x Intervention	$F_{(4, 36)} = 15.93$, p=.001	$F_{(4, 36)} = 59.34$, p=.001
	Genotype x Time x Intervention	$F_{(4, 36)} = 19.81$, p=.001	$F_{(4, 36)} = 24.42$, p=.001
Rotarod	Genotype	$F_{(1, 18)} = 25.27$, p=.001	$F_{(1, 18)} = 50.72$, p=.001
	Time	$F_{(2, 36)} = 2.33$, p=.112	$F_{(2, 36)} = 17.92$, p=.001
	Intervention	$F_{(2, 18)} = 0.19$, p=.825	$F_{(2,18)} = 3.075$, p=.071
	Genotype x Time	$F_{(2, 36)} = 0.27$, p=.766	$F_{(2, 36)} = 13.30$, p=.001
	Time x Intervention	$F_{(4, 36)} = 2.07$, p=.104	$F_{(4, 36)} = 7.57$, p=.001
	Genotype x Time x Intervention	$F_{(4, 36)} = 2.85$, p=.083	$F_{(4, 36)} = 5.00$, p=.003

DA levels are positively correlated with daily running wheels use

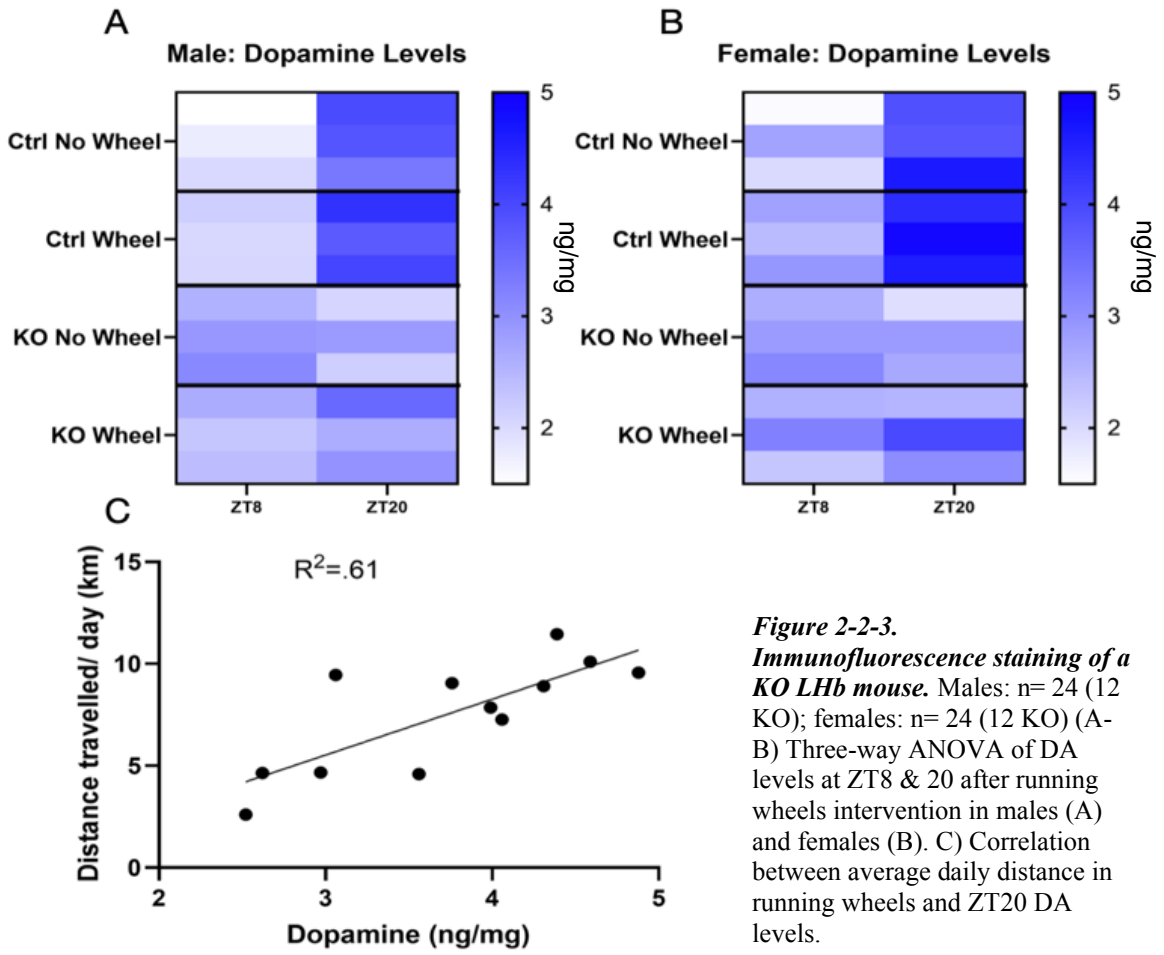


Figure 2-2-3. Immunofluorescence staining of a KO LHb mouse. Males: n= 24 (12 KO); females: n= 24 (12 KO) (A-B) Three-way ANOVA of DA levels at ZT8 & 20 after running wheels intervention in males (A) and females (B). C) Correlation between average daily distance in running wheels and ZT20 DA levels.

Liquid chromatography coupled mass spectrometry was used to measure striatal DA levels at ZT8 and ZT20 in the non-pharmacological intervention group (running wheels). Daytime DA levels in KO animals were normalized to similar levels as controls (Fig. 3A &B). Conversely, while DA levels in mice given running wheels were increased at night compared to the non-intervention group, these levels remained significantly lower than control mice levels (Fig. 3A &B). Notably, there was a significant positive correlation between average daily distance travelled in the running wheel and DA levels at ZT20. This indicates that as running wheel usage increases, so do DA levels (Fig. 3C). Together this indicates that, running wheels only marginally normalized DA levels, and the magnitude of effect was related to the level of wheel usage.

Motor interventions moderately normalized SN gene expression

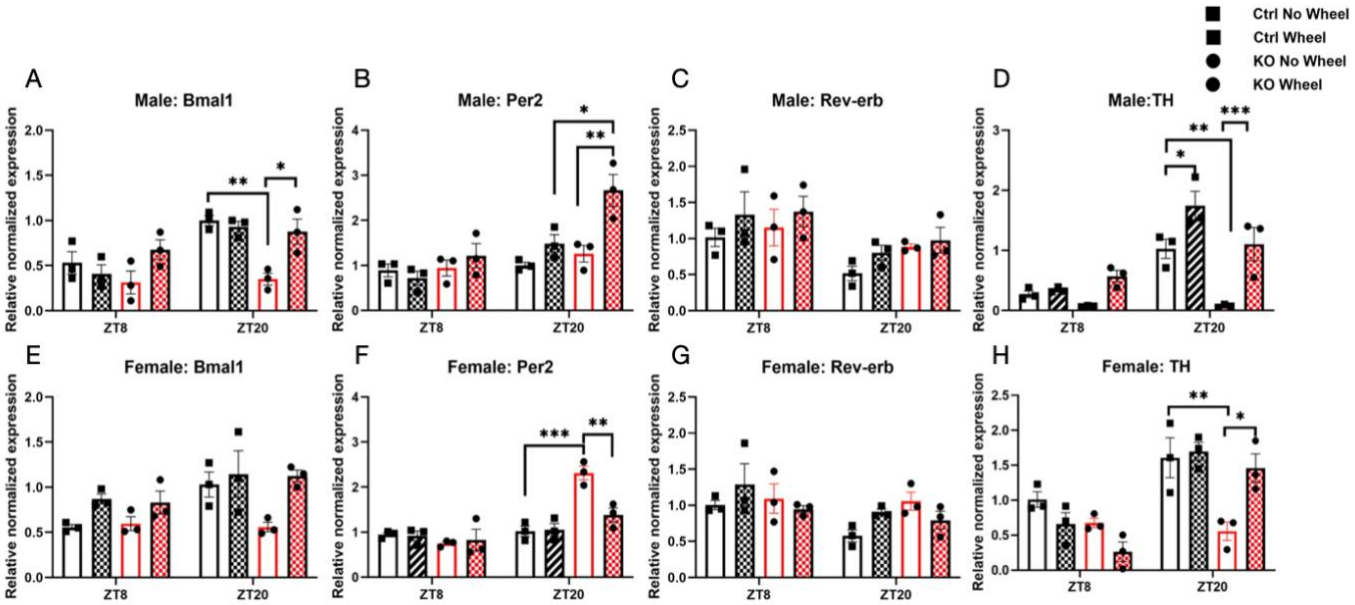


Figure 2-2-4. Substantia nigra gene expression in the non-pharmacological group. Males: n= 24 (12 KO); females: n= 24 (12 KO). 3-way ANOVAs were run for all tests. Analyses were separated by sex. (A & E) *Bmal1* levels in males (A) and females (E). (B & F) *Per2* levels in males (B) and females (F). (C & G) *Rev-erb* levels in males (C) and females (G). (D & H) *Tyrosine hydroxylase* levels in males (D) and females (H).
Notes. *= p<.05, **= p<.01, ***= p<.001

Table 2-2. Gene expression: Nonpharmacological group.

Target gene		Two-way ANOVA	
		Male	Female
Bmal1	Genotype	$F_{(1, 16)} = 16.30$, p=.001	$F_{(1, 16)} = 5.578$, p=.031
	Timepoint	$F_{(3, 16)} = 18.47$, p=.001	$F_{(3, 16)} = 26.00$, p=.001
	Genotype x Timepoint	$F_{(3, 16)} = 18.31$, p=.001	$F_{(3, 16)} = 4.161$, p=.023
Per 2	Genotype	$F_{(1, 16)} = 0.687$, p=.420	$F_{(1, 16)} = 7.905$, p=.013
	Timepoint	$F_{(3, 16)} = 20.33$, p=.001	$F_{(3, 16)} = 12.93$, p=.001
	Genotype x Timepoint	$F_{(3, 16)} = 6.232$, p=.005	$F_{(3, 16)} = 20.79$, p=.001
Cry1	Genotype	$F_{(1, 16)} = 1.255$, p=.279	$F_{(1, 16)} = 2.381$, p=.142
	Timepoint	$F_{(3, 16)} = 1.800$, p=.188	$F_{(3, 16)} = 2.298$, p=.117
	Genotype x Timepoint	$F_{(3, 16)} = 4.724$, p=.015	$F_{(3, 16)} = 2.381$, p=.710
TH	Genotype	$F_{(1, 16)} = 30.84$, p=.001	$F_{(1, 16)} = 22.51$, p=.001
	Timepoint	$F_{(3, 16)} = 32.98$, p=.001	$F_{(3, 16)} = 20.59$, p=.001
	Genotype x Timepoint	$F_{(3, 16)} = 10.42$, p=.001	$F_{(3, 16)} = 6.293$, p=.005
Reverb	Genotype	$F_{(1, 16)} = 2.282$, p=.150	$F_{(1, 16)} = 2.547$, p=.092
	Timepoint	$F_{(3, 16)} = 6.207$, p=005	$F_{(3, 16)} = 3.973$, p=.027
	Genotype x Timepoint	$F_{(3, 16)} = 1.533$, p=.244	$F_{(3, 16)} = 1.597$, p=.224

To decipher any impact these interventions may have had on SN functioning, real-time PCR gene expression was assessed on tissue collected after behavioural testing. In males, running wheels increased *Per2* levels at ZT20 in KO mice to far exceed control levels (Fig. 4B, Table 2). Whereas, in females, these levels were normalized to control levels (Fig. 4F, Table 3). Interestingly, running wheels use resulted in normalized *Bmal1*, *TH,* and *Cry1* levels in both male and female KO mice (Fig. 4A, D, E, & H, Table 2).

In the pharmacological intervention group, *Per2* levels were increased after quinpirole injections in all groups (Fig. 5B & F, Table 3). Moreover, quinpirole injections resulted in normalized *Bmal1* and *TH* levels in KO females, but not males (Fig. 5A, D, E, & H, Table 3). Further, *Rev-erb* levels were normalized to control levels in both males and females (Fig. 5C & G, Table 3). As such, both quinpirole injections and running wheel interventions moderately normalize SN gene expression after disruption due to LHb *Bmal1* KO.

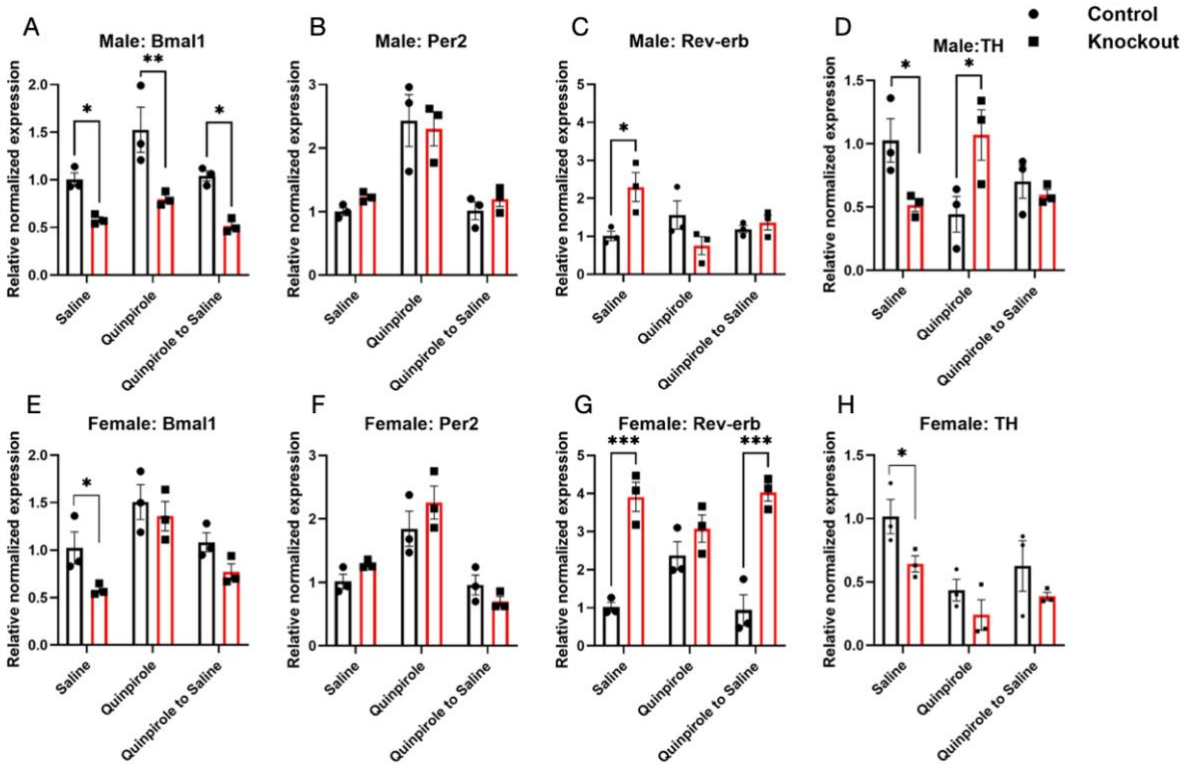


Figure 2-2-5. Substantia nigra gene expression in the Pharmacological intervention group. Males: n= 18 (9 KO); females: n= 18 (9 KO). 3-way ANOVAs were run for all tests. Analyses were separated by sex. (A & E) *Bmal1* levels in males (A) and females (E). (B & F) *Per2* levels in males (B) and females (F). (C & G) *Rev-erb* levels in males (C) and females (G). (D & H) *Tyrosine hydroxylase* levels in males (D) and females (H). Notes. *= p<.05, **= p<.01, ***= p<.001

Table 2-3. Gene expression: Pharmacological group.

Target gene		Two-way ANOVA Male	Female
Bmal1	Genotype	$F_{(1, 16)} = 16.30$, p=.001	$F_{(1, 16)} = 5.578$, p=.031
	Timepoint	$F_{(3, 16)} = 18.47$, p=.001	$F_{(3, 16)} = 26.00$, p=.001
	Genotype x Timepoint	$F_{(3, 16)} = 18.31$, p=.001	$F_{(3, 16)} = 4.161$, p=.023
Per 2	Genotype	$F_{(1, 16)} = 0.687$, p=.420	$F_{(1, 16)} = 7.905$, p=.013
	Timepoint	$F_{(3, 16)} = 20.33$, p=.001	$F_{(3, 16)} = 12.93$, p=.001
	Genotype x Timepoint	$F_{(3, 16)} = 6.232$, p=.005	$F_{(3, 16)} = 20.79$, p=.001
Cry1	Genotype	$F_{(1, 16)} = 1.255$, p=.279	$F_{(1, 16)} = 2.381$, p=.142
	Timepoint	$F_{(3, 16)} = 1.800$, p=.188	$F_{(3, 16)} = 2.298$, p=.117
	Genotype x Timepoint	$F_{(3, 16)} = 4.724$, p=.015	$F_{(3, 16)} = 2.381$, p=.710
TH	Genotype	$F_{(1, 16)} = 30.84$, p=.001	$F_{(1, 16)} = 22.51$, p=.001
	Timepoint	$F_{(3, 16)} = 32.98$, p=.001	$F_{(3, 16)} = 20.59$, p=.001
	Genotype x Timepoint	$F_{(3, 16)} = 10.42$, p=.001	$F_{(3, 16)} = 6.293$, p=.005
Reverb	Genotype	$F_{(1, 16)} = 2.282$, p=.150	$F_{(1, 16)} = 2.547$, p=.092
	Timepoint	$F_{(3, 16)} = 6.207$, p=005	$F_{(3, 16)} = 3.973$, p=.027
	Genotype x Timepoint	$F_{(3, 16)} = 1.533$, p=.244	$F_{(3, 16)} = 1.597$, p=.224

Discussion

The bidirectional relationship between the circadian and DAergic systems has been well established (Chung et al., 2014; Hampp et al., 2008; Hood et al., 2010; Imbesi et al., 2009; Mcclung et al., 2005). Furthermore, it is clear that rhythmic DA is essential for DS clock functioning, and the striatal clock impacts motor performance. Less understood however, are the mechanism responsible for the rhythmic release of DA into the DS. Previous work found that DAergic synthesizing agents display daily oscillations due to clock gene control in the SN (Chung et al., 2014; Hampp et al., 2008). However, the SN does not have the capacity for self-sustained rhythms, does not receive light information, nor does it receive direct input from the SCN clock. It does however receive direct and indirect rhythmic signals from the clock in the LHb. As such, we previously aimed to understand the importance of the LHb clock in nigrostriatal functioning. After targeted deletion of *Bmal1,* mice demonstrated significant motor deficits, altered SN functioning, and blunted striatal DA rhythms, indicating that the *Bmal1* in the LHb clock acts by indirectly regulating DS DA.

As such, the purpose of the current study was to assess the possibility of rescuing this motor phenotype. We did so by using two classes of interventions: pharmacological or non-pharmacological. Our pharmacological intervention employed timed daily injections of the D2

agonist, quinpirole. Quinpirole acts by increasing the binding potential of D2 receptors, thereby stimulating the indirect pathway in the striatum. Moreover, quinpirole was previously found to restore motor functioning and *Per2* rhythms after alterations caused by 6-OHDA lesions to the medium forebrain bundle (Hood et al., 2010) and influence *Per1* and *Clock* functioning (Imbesi et al., 2009). Thus, we hypothesized that daily timed injections of quinpirole, given in the middle of the dark phase, may be able to restore DA rhythms altered by loss of LHb *Bmal1*.

Free-access to running wheels was utilized as the non-pharmacological intervention. Running wheels are well evidenced to feedback to the brain's clock and are capable of entraining rhythms (Bilu et al., 2022; Edgar et al., 1991; Edgar & Dement, 1991). Moreover, running wheel usage in mice has the added benefit of increasing striatal DA levels (Bastioli et al. 2021). Thus, both interventions influence circadian and DAergic functioning which were altered after *Bmal1* KO.

To assess the efficacy of these interventions, baseline motor measurements were taken, and mice were reassessed at either 2- or 4- week intervals. Overall, there was general improvement in motor functioning after both interventions, but neither completely restored altered molecular mechanisms.

Quinpirole injections improved rotarod functioning in a sex-dependent manner

In the present study, the pole test and rotarod were used to assess motor performance. The pole test was designed as a Parkinson's-like motor test, used as a tool for detecting striatal DA deficits (Ogawa et al., 1985; Sedelis et al., 2001). However, rotarod performance in mice with altered striatal DA has varied. Some have found no deficits on rotarod performance (Sedelis et al., 2001), while others reported impairments, especially at higher speeds (Akita et al., 2006; Monville et al., 2006; Rozas et al., 1998; Yin et al., 2009). Moreover, each test highlights different aspects of motor functioning: the pole test primarily assesses coordination, the rotarod assesses endurance. Thus, both assessments were used to ensure testing captured a comprehensive picture of motor functioning before and after interventions.

The results clearly demonstrate that both interventions improved coordination on the pole test. However, in the pharmacological intervention group, only females improved in rotarod performance. Sex differences in response to quinpirole injections have been previously noted. Female rats display higher locomotor activity (Frantz, 1999; Schindler & Carmona, 2002) and

increased striatal DA levels in response to quinpirole injections (Walker et al., 2006). As well, quinpirole led to increased sensitivity to DAergic stimuli in females (Calipari et al., 2017; Walker et al., 2006; Zachry et al., 2021). As such, females likely had a stronger response to the quinpirole injections, showing higher overall motor improvements.

Lower improvement on the rotarod may also be due to the cell-type specific nature of the agonist. Quinpirole was chosen because it is a D2 receptor agonist, and lower functioning of the indirect D2 pathway has been previously linked to striatal DA disorders (Dauer & Przedborski, 2003; Ramesh & Arachchige, 2023). However, it is unknown if changes in striatal DA functioning due to LHb *Bmal1* KO solely impact this system. Rather, these results suggest our KO likely impacts the D1 system to some degree as well, as previous evidence suggests that rotarod performance is linked to the D1 system functioning (Willuhn & Steiner, 2008). Thus, our direct targeting of D2 receptors through quinpirole injections would likely have a limited impact on this test.

Running wheels normalized motor functioning, but not DA levels

Results from gene expression analyses on SN tissue in KO mice receiving daily quinpirole injections were sex dependent: *Bmal1* was downregulated, and *TH* was upregulated in males, with the opposite pattern in females. That levels of *TH* and *Bmal1* remain in anti-phase with each aligns with previous findings in the midbrain (Guo et al., 2018). Notably, the opposite pattern in males and females demonstrates sex-dependent mechanistic result of daily quinpirole injections and likely contributes to the female-only rotarod improvement.

In the non-pharmacological intervention groups, running wheel access resulted in normalized levels of *Bmal1* and *TH* in both male and female KO mice. As with motor performance, males and females demonstrate similarly improved SN functioning in this intervention.

However, while there were no apparent sex differences in the non-pharmacological group, this doesn't indicate that the intervention fully rescued LHb *Bmal1* KO induced deficits. In fact, while there was an overall improvement in motor functioning, DA levels were not normalized at both timepoints. While levels at ZT8 were no longer as elevated as the home cage KO groups, levels at ZT20 remained significantly lower in KO mice after intervention. Thus, if

motor functioning appears to be rescued, but DA levels were not entirely normalized, then other systems must also be responsive to running wheel usage and influencing motor performance.

The striatum has strong serotoninergic (5-HT) inputs (Vertes, 1991), which act on both the cholinergic interneurons and directly on DAergic neurons. 5-HT inputs to striatal cholinergic neurons have been linked to voluntary motor movement and to motor dysfunction in Parkinson's disease (Kaneko et al., 2000; Raz et al., 2001). Moreover, running wheels have strong feedback on the 5-HT system (Greenwood et al., 2003, 2011). As such, while DA clearly plays an essential role in the motor deficits found previously, it does not function alone in moderating these behaviour.

Overall, both interventions had moderate success at rescuing deficits after a LHb *Bmal1* KO. That quinpirole injections only improved rotarod performance in female mice and that running wheel usage did not entirely restore DA levels highlights that motor functioning is neither cell-type nor pathway specific. Future research should look outside the DAergic system to create a more comprehensive picture of the systems involved in motor functioning. These findings pave the way for further research into targeted therapies that consider the intricate relationships between the circadian clock, neurotransmitter systems, and motor function, with attention to sex-specific responses.

Method

Subjects

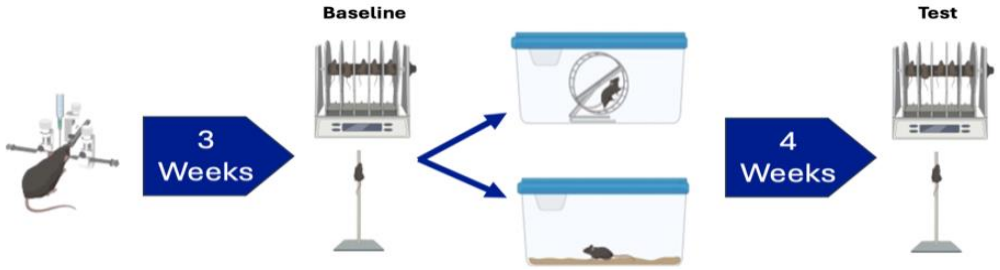
Adult male and female Bmal1 floxed mice (21 - 28 g) originally obtained from Jackson laboratories. 96 mice were used in total, 48 males and 48 females. Animals were divided into two groups, non-pharmacological and pharmacological. The non-pharmacological group consisted of 24 males (12 control/ 12 knockout) and 24 females (12 control/ 12 knockout). Pharmacological consisted of 24 males (12 control/ 12 knockout) and 24 females (12 control/ 12 knockout).

Male and Female mice were group-housed under a 12:12 light-dark (LD) cycle at 21 ± 2°C and 60% relative humidity with food and water available *ad libitum* prior to the stereotactic delivery of viral vectors at 12 – 16 weeks of age. The bedding of the cages was changed every week. Animals were housed individually after the surgery under the same housing conditions as described above unless stated otherwise.

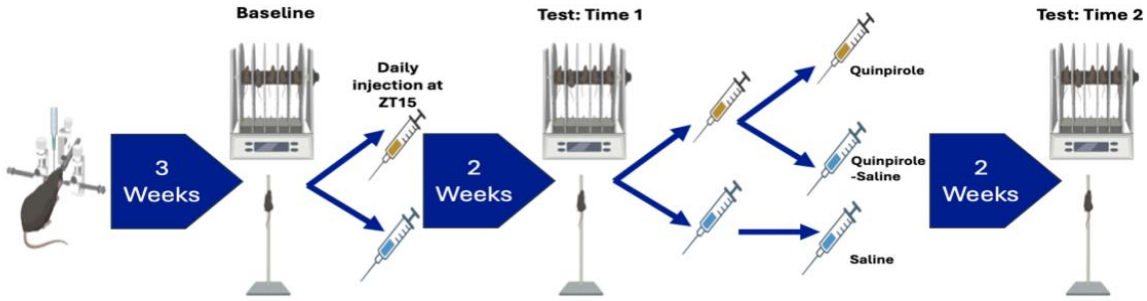
All experiments were conducted according to the guidelines and requirements of the Canadian Council on Animal Care (CCAC) and approved by the Concordia University ethics committee (AREC number 3000256).

Experimental timeline:

Nonpharmacological group



Pharmacological group



Stereotactic surgery

Mice were weighed and then anesthetized with a mix of ketamine and xylazine solution (0.1mL/20g) and placed in a stereotaxic apparatus (KOPF, Tujunga, CA). Using a 30-gauge needle, animals received 150nl bilateral microinjections of either the AAV-2/9-CAG-CRE-eGFP (1x10^12 vg/ml) or AAV-2/9-CAG-eGFP (1x10^12 vg/ml) viruses. The injector was inserted at a 0° angle into the LHb (AP: -1.65, ML: 0.4, and DV: -3.1). The injector was left in place for five minutes following the injection to optimize diffusion and then withdrawn completely. Mice were allowed to recover for three weeks before behavioural testing.

Pole Test

The pole test is common test to assess motor function in mice. Animals were habituated to the experimental room for 30 minutes prior to the test, which has been conducted between ZT8-ZT10. Mice were placed head-upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm) placed in an enclosure filled with bedding (5 cm) to cushion animals falling from the pole. Mice were trained on how to descend the pole before testing began. Mice were given 120 seconds to descend the pole, time was stopped upon reaching the enclosure surface. Scores were averaged over three trails, with 30 seconds intertrial intervals. If mice failed to turn downward and descend the pole or if the mouse fell, time was taken as 120 second (default value). The criteria assessed includes the proportion of those who were able to complete the turn and the total time to descend to the ground over three trials. Animals were video recorded (Samsung Galaxy A5 2017), and the videos were scored by an experienced researcher blind to the experimental conditions using a stopwatch.

Rotarod

A custom-made, fixed-speed rotarod (FSRR) was used to further assess motor coordination in experimental and control mice of both sexes between ZT6–8. In brief, the rotarod was composed of a rod (ø 5 cm) mounted 19.5 cm above the base of the device. The base was separated into 6 individual compartments equipped with a soft foam pad to cushion mice falling off the rod. To prevent mice from leaving the rod, two flanges (ø 20 cm) separated a 9.3 cm interspace on the rod. The rod was driven by an electric motor, and a gearbox was used to set the desired speed. On the day of the experiment, animals underwent a training session on the rotarod between ZT2–4 to familiarize themselves with the apparatus. For this, animals were gently grabbed by their tail and carefully placed on the rod turning at 2 rpm for 5 min. Animals falling from the rod were always put back on the rod throughout the entire training period. After the training, animals were transferred back to their home cages. Mice underwent testing between ZT6–8 the same day. While being videorecorded, animals were positioned on the turning rod as described above and left on the rod for a 1-min trial period, except that they were not put back on the rod when falling from it within the trial. If a mouse fell off the rod due to poor placement by the experimenter, the trial was not counted and repeated after a short resting time. Mice were gently transferred into the compartment at the base of the Rotarod at the end of each trial and left

for a 30 s resting period. Each trial was repeated two more times before the rod was set to a higher speed. The starting speed was 2 rounds per minute (rpm), which was gradually increased to 4, 8 and 12 rpm. If animals failed to stay on the rod for the 1-min period at all 3 trials, they were not further tested at higher speeds.

DA Receptor Agonist Administration

After baseline testing, male and female mice were randomly assigned into two groups (control group vs. test group). After which mice were given daily intraperitoneal injections (10 ml/kg body weight) of either saline or the D2 receptor agonist quinpirole (1 mg/kg body weight, Cayman Chemical, Ann Arbor, MI, United States). Injections were given three hours into the dark phase, at ZT15. The procedure was repeated every day for two weeks, after which motor functions were tested again. To assess the longevity of these injections, after behavioural testing, half of the mice receiving quinpirole continued to receive daily quinpirole injections (quinpirole group), while the other half began receiving daily saline injections (quinpirole-saline group). After two weeks mice were again tested on the pole test and rotarod and then euthanized 2 hours after final injection ZT17.

Running wheel analysis

All mice underwent baseline motor testing for the pole and rotarod tests and were then randomly assigned to either a running wheel cage or standard home cage. After 4 weeks, mice were reassessed on motor tests. After testing, mice were euthanized at ZT8 or ZT20 and DS and SN tissue was collected. Wheel running was recorded and analyzed using Clocklab 6 (Actimetrics, Wilmette, IL, USA). Average daily running wheel counts given as revolution per minute (RPM) was assessed from summed activity counts, and daily distance travelled was calculated using the formula: Distance = RPM x wheel circumference.

Tissue collection

Serial coronal sections (100 µm thick) were obtained using a Cryostat (Microm HM 505 E). Tissue punches of the CP (1.5 mm in diameter) and the SN (1 mm in diameter) were collected from each hemisphere in accordance with the mouse brain atlas (Paxinos & Franklin, 2003).

LCMS

Frozen brain tissue samples were thawed on ice and homogenized in 150ul of 80% methanol with 2uM concentration of isotope-labeled DA.

The mixture was centrifuged at 4 °C, 14,000 rpm, for 20 min. The supernatant was transferred into a new tube and dried under nitrogen vacuum on ice. The residue was reconstituted in 1000ul of ultrapure water along with a final concentration of 0.1M 3NT standard.

2-(3,4-Dihydroxyphenyl)ethyl-1,1,2,2-d4-amine HCl (D4-DA, C/D/N ISOTOPES) was used as an internal standard in the extraction solution. The DA (DA) standards were prepared in a pooled extracts of mouse brain tissues at concentrations of 0.05, 0.1, 0.5 and 1 μM. The calibration curve was plotted using the ratios of peak areas of DA to D4-DA and the slope was used to calculate DA concentrations in samples.

LC-MS analyses were performed on an Agilent 1100 LC system coupled to a Thermo LTQ Orbitrap Velos mass spectrometer equipped with a heated electrospray ion source at positive mode. A Waters Atlantis dC18 column (100 x 2.1 mm, 3 μ particle diameter) was used and target compounds were eluted using a 18-min gradient at a flow rate of 250 μL/min with mobile phase A (99.9% water and 0.1% formic acid (FA)) and B (99.9% Acetonitrile and 0.1% FA). The gradient started at 2% B and held for 4 min, linear gradients were achieved to 90% B at 6 min, then followed by isocratic with 90% B for 2 min and with 2% B for 9 min. 5 μL of each sample was injected for the LC-MS analysis. MS spectra (*m/z* 100-250) were acquired in the Orbitrap at a resolution of 60000, DA and D4-DA were targeted at *m/z* 154.0863 and 158.1114 at retention time of 2.5 min. Peak area values were extracted using Thermo XCalibur software (v2.2 SP1.48).

Real-time PCR

A standard Trizol extraction protocol was used for total RNA isolation according to the manufacturer's instructions (Ambion, Carlsbad, CA). Total RNA yield was measured using spectrophotometry, and RNA integrity was assessed by the "bleach gel" method (Aranda et al., 2012). cDNA was synthesized from 1 ug of RNA using the iScript™ Reverse Transcription Supermix (Biorad, Hercules, CA, USA). Beside the standard cDNA samples, a no reverse transcriptase (no-RT) control was prepared. SYBR green based quantitative real-time PCR was

used to analyze gene expression. 10 ul reactions containing SsoAdvanced Universal SYBR® Green Supermix (Biorad, Hercules, CA, USA) and 300 uM of the respective forward and reverse primers were amplified using a CFX96^{TM} Real-Time PCR system (Biorad, Hercules, CA, USA).

The CFX Maestro qPCR Analysis Software (Biorad, Hercules, CA, USA) was used to calculate relative changes in gene expression between samples by the delta-delta Ct (ΔΔCt) method (Pfaffl, 2001).

Author contributions

K.S., S.A., and C.G. conceived and designed the study; C.G., N.B., and H.J. performed the experiments; C.G., K.S., and S.A. analyzed and interpreted the data, and wrote the manuscript; S.A. and K.S. Supervision.

Competing interests

The authors declare no competing interests.

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Chapter 3

Bmal1 in the lateral habenula has a sex dependent effect on alcohol intake

Cassandra Goldfarb, Vanessa Hasenhundl, Arianne Menasce, Shimon Amir, Konrad Schöttner

Abstract

The circadian clock regulates most aspects of mammalian physiology and behaviour, including alcohol drinking behaviour. Disrupted circadian clock function via deletion of clock genes along the mesolimbic dopamine (DA) pathway has been linked to altered patterns of alcohol drinking behaviour. The lateral habenula (LHb), an epithalamic structure that house a semi-autonomous circadian clock, is a negative regulator of the mesolimbic DA system and of alcohol consumption. To study the role of the LHb circadian clock in alcohol consumption, we knocked out the core clock gene, *Bmal1* specifically in the LHb and examined the impact on various alcohol drinking paradigms and affective behaviours in male and female mice. Our findings demonstrate that *Bmal1* deletion in the LHb leads to sex-specific alterations in alcohol consumption. Male knockout mice exhibited increased voluntary alcohol intake and elevated alcohol binge drinking compared to controls. Conversely, female knockouts showed a marginal decrease in voluntary intake, significantly reduced consumption of an aversive alcohol solution, and lower post abstinence, relapse-like drinking. These results indicate that *Bmal1* in the LHb exerts a repressive effect on alcohol intake in males, while it inhibits intake under certain aversive conditions in females. Interestingly, *Bmal1* deletion did not significantly affect anxiety-like or depressive-like behaviours, suggesting that the habenular clock's role in alcohol consumption is independent of affective state. These findings mark *Bmal1* in the LHb as a novel sexually dimorphic regulator of alcohol consumption in mice. Potential mechanisms involving the circadian modulation of DA and serotonin signaling pathways by the LHb clock is discussed.

Key words

Habenula, circadian clock, Bmal1, alcohol, sex differences, mice

Introduction

Circadian rhythms are regulated by cellular clocks comprising a small set of so-called clock genes (Partch et al., 2014; Takahashi, 2017). These genes drive transcription and translation feedback loops that are at the core of the ~24 rhythm generated by the clock. In mouse models, disruption or complete loss of function in different clock genes is associated with abnormal or total loss of circadian rhythmicity and in a range of physiological, metabolic, and behavioural disturbances (Bunger et al., 2000; King et al., 1997; Kume et al., 1999). In recent years, evidence has emerged linking clock genes, including *Bmal1, Clock, Per2, and Rev-erba* with alcohol drinking behaviour and alcohol use disorder (AUD). For example, polymorphisms in *Bmal1* and *Per2* have been linked to abnormal alcohol consumption, suggesting a clock gene based predisposition for developing AUD (Kovanen et al., 2010; Partonen, 2015; Valenzuela et al., 2016). In mice, constitutive knockouts of *Per2* and *Clock*, as well as selective suppression of *Clock* in the nucleus accumbens (NAc), can increase alcohol intake (Gamsby et al., 2013; Kirkpatrick et al., 2009; Rizk et al., 2022; Spanagel et al., 2005). Conversely, global knockout of *Rev-erba* decreases alcohol consumption in mice (Al-Sabagh et al., 2022), highlighting distinct roles for different clock genes in regulating alcohol drinking behaviour. More recently, we found that conditional deletion of *Bmal1* from medium spiny neurons (MSNs) throughout the striatum augments voluntary alcohol consumption and preference in male mice and conversely, repress intake and preference in females (de Zavalia et al., 2021b). Interestingly, deletion of *Bmal1* only in the NAc augmented alcohol intake in both sexes (Herrera et al., 2023), indicating that *Bmal1* influences alcohol consumption via both shared, sex-independent mechanisms within the NAc and distinct, sex-specific mechanisms likely in the dorsal striatum.

It is unknown whether the sex dependent and independent effects of *Bmal1* on alcohol consumption are specific to the striatum or whether they extend to other brain regions implicated in the control of alcohol drinking behaviour. In the present study we focused on the lateral habenula (LHb), a major negative regulator of the midbrain dopaminergic neurons that supply the striatum (Ji & Shepard, 2007; Matsumoto & Hikosaka, 2007; Salaberry & Mendoza, 2016), and a key player in aversive conditioning and alcohol drinking behaviour (Fu et al., 2017; Mondoloni et al., 2022; Salaberry & Mendoza, 2016; Zuo et al., 2014). Notably, the LHb houses a semi-autonomous circadian clock (Baño-otálora & Piggins, 2017; Guilding et al., 2010a;

Mendoza, 2017b; Pradel et al., 2022b; Sakhi, Belle, et al., 2014), suggesting a potential influence on alcohol consumption via indirect circadian control of DA signaling in the striatum.

To study the role of the LHb clock in alcohol consumption, we investigated the effect of conditional deletion of *Bmal1* in the LHb on daily intermittent alcohol exposure (IAE), aversion-resistant consumption, reintroduction after forced abstinence, and alcohol binge drinking in male and female mice. Given the LHb's involvement in affective behaviours (Hikosaka et al., 2008; Mendoza, 2017b; Yang et al., 2018), depressive- and anxiety-like behaviours were assessed to identify potential interactions between *Bmal1*-mediated changes in alcohol consumption and affective state.

Results

Knockout of *Bmal1* in the LHb

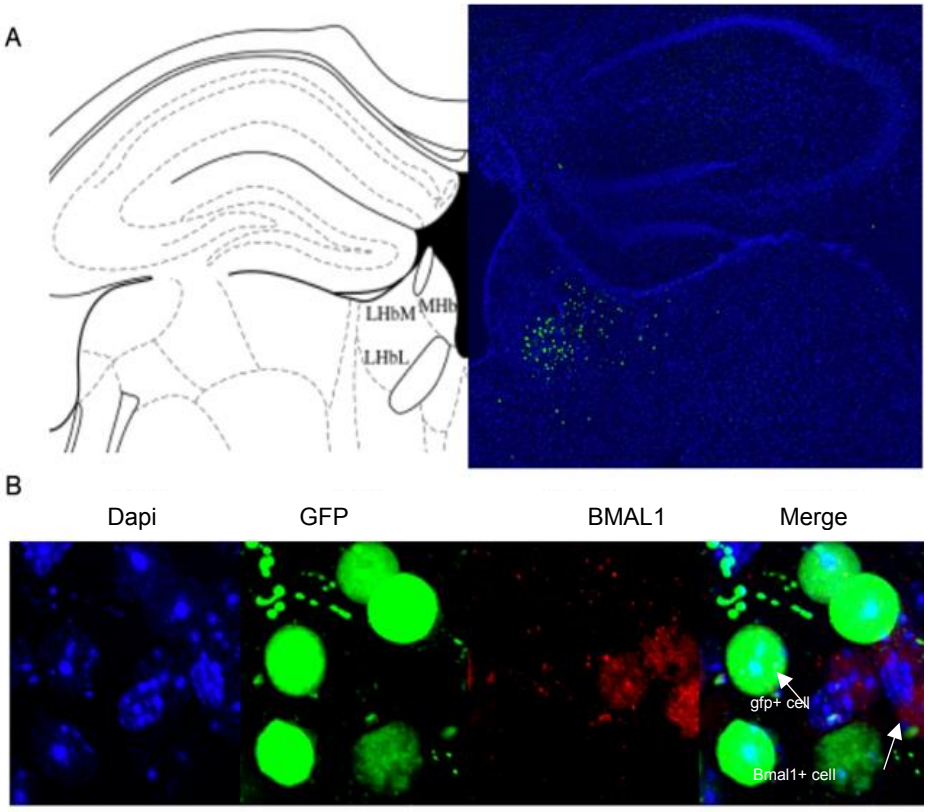


Figure 3-1. Representative immunofluorescence staining of viral expression in the LHb of mice receiving stereotaxic injections of AAV-CAG-Cre-EGFP viral vector. A) GFP staining is centralized in the LHb. B) 60x representative image of Dapi (blue), GFP (green), and BMAL1 (red) immunofluorescence staining in LHb tissue of a knockout mouse. Arrows point to either gfp positive cell or a BMAL1 positive cell, the same arrow placements in the merged square demonstrate no overlap between gfp and BMAL1.

Bmal1 floxed male and female mice received bilateral intra-LHb infusions of adeno associated virus (AAV) expressing Cre-recombinase and eGFP (KO) or AAV expressing eGFP only (CTR). Analysis of GFP and Cre expressing cells from histologically validated surgeries carried out at the end of the study revealed that ~70% of cells in the LHb of

experimental animals expressed Cre/eGFP (Fig. 1A). Immunofluorescence images confirm BMAL1 deletion in the habenula of KO mice (Fig. 1, B). Animals with low numbers of infected cells (less than 70% of cells infected) or off target infection were excluded from analyses.

Bmal1 deletion in the LHb has a marginal effect on anxiety and depressive like behaviour.

Tests for anxiety- and depressive- like behaviours began three weeks after surgery using the elevated plus maze test (EPM), open field test (OFT), and the sucrose preference test (SPT).

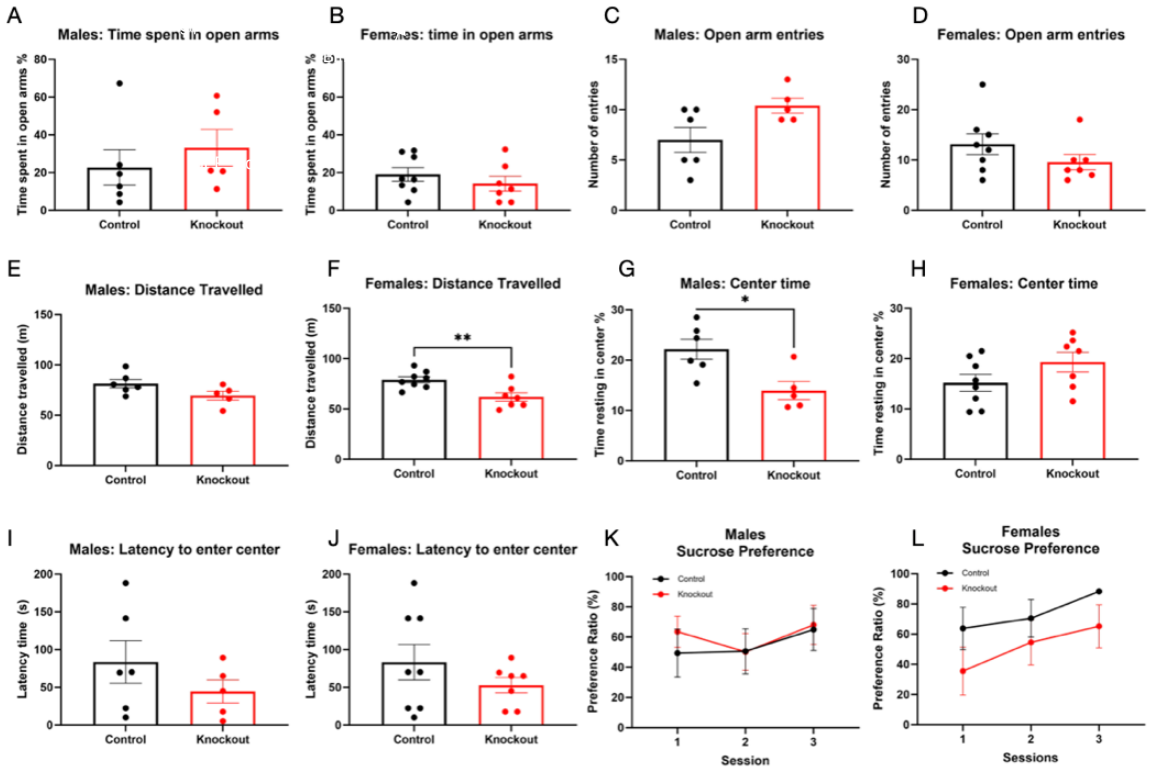


Figure 3-3-2. LHb Bmal1 does not meaningfully impact affective behaviours. Males: n = 11 (5 KO); females: n = 15 (7 KO). Independent sample T-tests were run for all tests, excluding sucrose preference test. Analyses were separated by sex. (A-B) Elevated plus maze, percentage of time spent in open arms; males (A) and females (B). (C-D) Elevated plus maze, number of entries into the open arms; males (C) and females (D). (E-F) Open-field test, distance travelled; males (E) and females **p < .01 (F). (G-H) Open-field test, time resting in the center of the open-field; males *p < .05 (G) and females (H). (I-J) Open-field test, latency to enter the center of the open-field; males (I) and females (J) (K-L) Two-way ANOVA for sucrose preference across three days for males (K) and females (L).

Deletion of *Bmal1* in the LHb had no effect on standard measures of anxiety-like behaviours in the EPM in either males or females (Fig. 2A, B, C, & D, Table 1.). In the OFT, knockout females travel significantly less compared to controls (Fig. 2F, Table 1.), whereas male knockouts spent significantly less time in the center of the field compared to controls (Fig. 2G, Table 1.). The knockout had no effect on the latency to enter the center of the field in either

males or females (Fig. 2I & J, Table 1). Lastly, in both males and females, deletion of *Bmal1* had no effect on sucrose preference, a test for anhedonia-like behaviour in mice (Fig. 2K & L, Table 1). Thus, deletion of *Bmal1* in the LHb does not significantly affect measures of anxiety and depressive like behaviours in the EPM and SPT and only marginally influences behavioural measures in the OFT, suggesting that the LHb circadian clock is not critically involved in the control of affective state.

Table 3-1. Measures of affective behaviour.

Test	Males	Females
EPM: time in open arms	$t_{(9)} = 0.77$, $p = .975$, n= .000	$t_{(13)} = 0.92$, $p = .373$, n= .061
EPM: open arms entries	$t_{(9)} = 2.23$, $p = .053$, n= .355	$t_{(13)} = 1.35$, $p = .200$, n= .123
OFT: distance travelled	$t_{(9)} = 1.93$, $p = .085$, n= .294	$t_{(13)} = 3.35$, $p = .005$, n= .463
OFT: time in center	$t_{(9)} = 2.99$, $p = .015$, n= .499	$t_{(13)} = 1.61$, $p = .131$, n= .166
OFT: latency to enter	$t_{(9)} = 1.10$, $p = .299$, n= .119	$t_{(13)} = 1.13$, $p = .279$, n= .089
SPT	$t_{(9)} = 0.32$, $p = .757$, n=.011	$t_{(13)} = 1.075$, $p = .302$, n= .080

Sex dependent effect of *Bmal1* deletion in the habenula on alcohol consumption

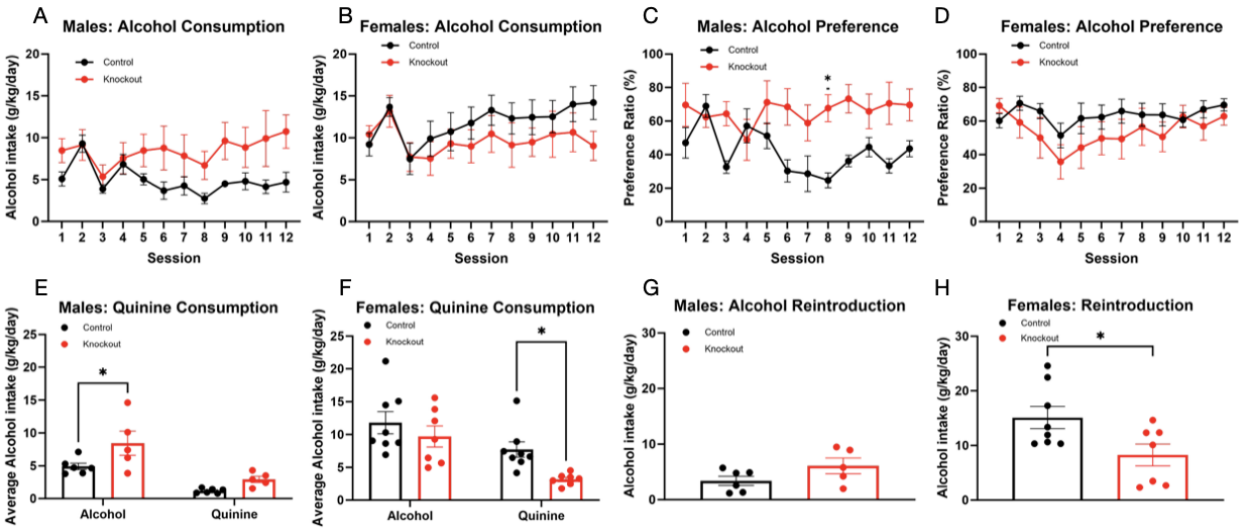


Figure 3-3-3. LHb Bmal1 KO increased drinking in males, and attenuated levels in females. Males: n= 11 (5 KO); females: n= 15 (7 KO). Daily alcohol intake for males (A) and females (B) across 12 sessions of two-bottle choice intermittent exposure (IAE) paradigm. Daily alcohol preference for males (C) and females (D) calculated as ml of alcohol solution consumed/total fluid intake across 12 sessions of IAE. Alcohol intake for males (E) and females *p < .05 (F) across three sessions of IAE after the addition of quinine. Alcohol intake for males (G) and females *p < .05 (H) after a period of forced abstinence.

The effect of *Bmal1* deletion on voluntary alcohol consumption was studied using an intermittent exposure two-bottle choice paradigm (IAE) in which mice had free access to one bottle of 15% ethanol solution and one bottle of tap water every other day for a total of 12 daily sessions. The results demonstrate sex specific effects of LHb *Bmal1* deletion on voluntary alcohol consumption. While male knockouts gradually increased their alcohol intake and preference over the 12 sessions relative to controls (Fig. 3A & C, Table 2), a marginal, statistically insignificant negative effect on consumption and preference was observed in females (Fig. 3B & D, Table 2).

Table 3-2. Alcohol drinking results.

Alcohol paradigm		Male	Female
IAE Consumption	Genotype	$F_{(1, 9)} = 4.10$, p=.074	$F_{(1, 13)} = 0.5040$, p=.489
	Session	$F_{(11, 9)} = 3.184$, p=.001	$F_{(11, 154)} = 3.844$, p=.001
	Genotype x Session	$F_{(11, 9)} = 2.010$, p=.035	$F_{(11, 154)} = 1.22$, p=.278
IAE Preference	Genotype	$F_{(1, 9)} = 7.006$, p=.027	$F_{(1, 13)} = 0.748$, p=.402
	Session	$F_{(11, 9)} = 2.418$, p=.059	$F_{(11, 154)} = 2.258$, p=.058
	Genotype x Session	$F_{(11, 9)} = 4.179$, p=.001	$F_{(11, 154)} = 1.047$, p=.409
Quinine	Genotype	$F_{(1, 9)} = 31.37$, p=.001	$F_{(1, 13)} = 14.30$, p=.002
	Session	$F_{(11, 9)} = 5.760$, p=.012	$F_{(11, 154)} = 0.390$, p=.680
	Genotype x Session	$F_{(11, 9)} = 2.762$, p=.098	$F_{(11, 154)} = 2.530$, p=.098
IAE average VS. Quinine	Genotype	$F_{(1, 18)} = 8.70$, p=.009	$F_{(1, 26)} = 6.189$, p=.020
	Substance	$F_{(11, 9)} = 26.19$, p=.001	$F_{(1, 26)} = 15.76$, p=.001
	Genotype x Substance	$F_{(11, 9)} = 0.938$, p=.345	$F_{(1, 26)} = 0.831$, p=.370
Reintroduction		$t_{(9)} = 1.73$, p=.118, $\eta^2 = .25$	$t_{(13)} = 2.39$, p=.032, $\eta^2 = .31$
DID	Genotype	$F_{(1, 6)} = 2.353$, p=.176	$F_{(1, 7)} = 1.425$, p=.272
	Session	$F_{(3, 18)} = 25.1$, p=.001	$F_{(3,21)} = 40.48$, p=.001
	Genotype x Session	$F_{(3, 18)} = 3.854$, p=.027	$F_{(3,21)} = 1.881$, p=.164

Two days after the last IAE session, all mice were given access to one bottle of 15% alcohol solution containing bitter quinine (250 uM) and one bottle of water for three consecutive days. Here, we aimed to assess if the effects of LHb *Bmal1* KO on voluntary alcohol drinking extend to the consumption of an aversive alcohol solution. The addition of quinine to the alcohol solution suppressed the levels of consumption in males compared to the average levels seen in the previous 12 intermittent drinking sessions. However, despite the overall decrease in consumption, KO males consumed significantly more of the bitter alcohol solution compared to controls (Fig. 3E, Table 2) whereas female knockouts consumed significantly less of the bitter alcohol solution relative to controls (Fig 3F, Table 2). Thus, in males, knocking out *Bmal1* in the LHb augments intake of both normal and aversive alcohol solutions. In contrast LHb knockout in females has little effect on consumption of a normal alcohol solution but represses consumption of a bitter alcohol solution.

In the last phase of this experiment, we assessed the effect of LHb *Bmal1* deletion on alcohol consumption following a period of forced abstinence, a test of relapse-like drinking behaviour. In males, both control and knockout mice consumed less alcohol during the 24-hours drinking test compared with the average levels seen across the 12 IAE sessions, and no significant differences in intake were noted between knockouts and controls (Fig. 3G, Table 2). In contrast, knockout females consumed significantly less alcohol than controls during the 24 hours test (Fig. 3H, Table 2), pointing to a female specific inhibitory effect on drinking after forced abstinence.

Male specific effect of *Bmal1* deletion in the habenula on alcohol binge drinking

In a separate experiment, we assessed the impact of *Bmal1* deletion in the LHb on alcohol binge drinking using the "drinking in the dark paradigm" as an experimental model (Thiele & Navarro, 2014). For this experiment, alcohol naïve knockout and control mice received a single bottle of 20% ethanol for two hours on three consecutive nights, starting two hours after lights off (ZT14-16), and for four hours on the fourth, "binge drinking" night (ZT14-18). As expected, consumption of the 20% alcohol solution increased significantly across the four sessions in all mice, regardless of sex or genotype (Fig. 4, Table 2). On the final four-hour session, male knockout mice drank nearly twice as much as controls (Fig. 4A & C, Table 2), demonstrating significant augmentation of binge drinking. In contrast, deletion of LHb *Bmal1* in females did

not significantly influence alcohol consumption during the test (Fig. 4B &D, Table 2), indicating a male-specific effect of a LHb *Bmal1* KO on binge drinking.

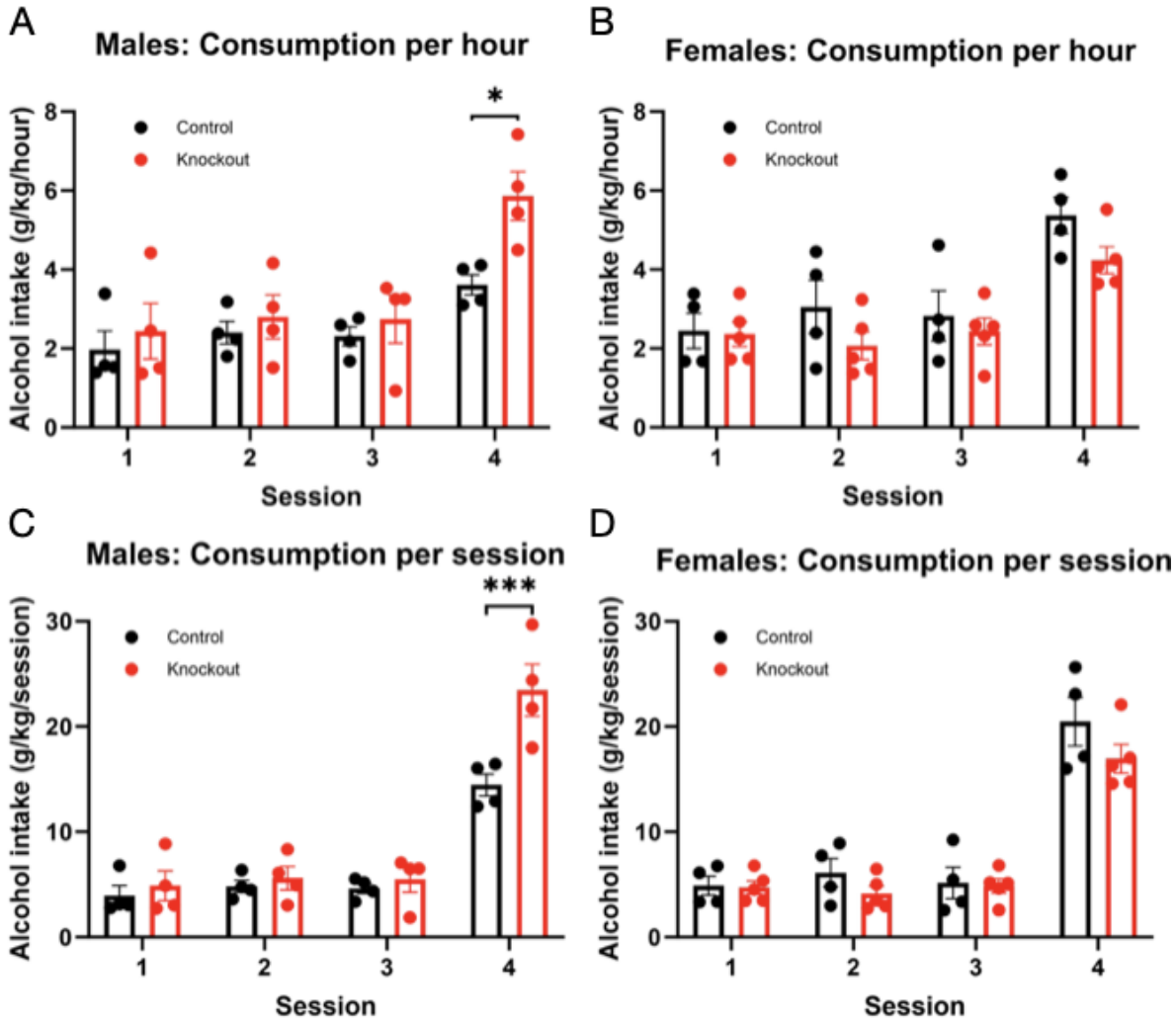


Figure 3-3-4. Male KO had a higher intake than controls. Males: n= 8 (4 KO); females: n= 9 (5 KO). (A) Male and (B) female "drinking in the dark" alcohol intake of control and knockout mice, normalized to hours of drinking. Per session alcohol consumption for males (C) and females (D). Sessions 1-3: 2 hours access to a 20% alcohol bottle; session 4: 4 hours access to a 20% alcohol bottle. Notes. * p<.05, *** p<.001

Discussion

This study identifies the LHb as a novel site of sex-specific effects of *Bmal1* on alcohol consumption in mice. In males, deletion of *Bmal1* in the LHb gradually augmented voluntary alcohol intake in the two-bottle choice test, attenuated the decrease in consumption of a bitter alcohol solution, and amplified binge-like alcohol consumption. In females, the same deletion did not significantly affect alcohol consumption in the two-bottle choice and binge drinking tests,

but it suppressed drinking of a bitter alcohol solution and attenuated relapse-like intake following a period of forced abstinence. These results suggest that the LHb circadian clock or other *Bmal1*-dependent mechanisms normally repress alcohol consumption in males and upregulate intake in females, under certain conditions. Furthermore, the findings support the hypothesis that *Bmal1*-dependent mechanisms across different brain regions contribute to sex differences in alcohol drinking in mice (de Zavalia et al., 2021).

LHb deletion on *Bmal1* did not affect anxiety and depressive-like behaviours

Notably, the deletion of *Bmal1* in the LHb did not affect anxiety and depressive-like behaviours in the EPM and SPT, and only marginally influenced anxiety measures in the OFT. These findings mirror previous results in mice with *Bmal1* deletion in the striatum or only in the NAc (de Zavalia et al., 2021b; Herrera et al., 2023; Schoettner et al., 2022), suggesting that affective state alone is not a significant determinant of the drinking phenotypes associated with *Bmal1* deletion in these regions. Interestingly, several clock genes have been associated with affective state in animal models and in humans (Benedetti et al., 2003; Chung et al., 2014; De Bundel et al., 2013; Mansour et al., 2006; Roybal et al., 2007; Russell et al., 2021; Schnell et al., 2015; Serretti et al., 2005; C. Zhao & Gammie, 2018), and anxiety and mood disorders commonly occur with alcohol use disorder, and the co-occurrence is associated with increased severity of both (McHugh & Weiss, 2019). Our present and previous studies suggest that any links between clock genes, affective state, and alcohol drinking behaviour are likely mediated outside the LHb-striatum circuit.

Neural mechanisms of LHb functioning

The neural mechanisms that mediate the effect of *Bmal1* in the LHb on alcohol consumption remains to be determined. One possibility is that loss of *Bmal1* disrupts neural communication between the LHb and the midbrain DA nuclei, altering DA synthesis and release in the striatum. Another possibility is that loss of *Bmal1*in the LHb affects the release of serotonin (5-HT) from the neurons of the raphe nuclei which, like midbrain DA neurons, have been implicated in alcohol drinking behaviour (Lovinger, 1997; Lovinger & Alvarez, 2017; Metzger et al., 2017; Sari et al., 2011). In support of the DA hypothesis, the clock in the LHb drives temporal variations in local expression of clock genes and neural activity (Guilding et al.,

2010a), and it plays a key role in the control of rhythmic DA synthesis and release from the neurons in the VTA and SN (Ji & Shepard, 2007; Langel et al., 2018; Matsumoto & Hikosaka, 2007). DAergic release from these neurons to the striatal regions has been implicated in the circadian control of striatal clock gene expression and in the regulation of alcohol drinking behaviour (Korshunov et al., 2017; McClung, 2007; Parekh et al., 2015; Tang et al., 2022; Verwey et al., 2016; Hood et al, 2010; Gravotta et al., 2011). Previous work indicates that a *Bmal1* knockout in both the whole striatum and the NAc specifically altered drinking behaviour (de Zavalia et al., 2021; Herrera et al., 2023). Thus, a molecular circadian clock in the LHb has the potential to indirectly influence striatal DA signaling, striatal clock gene expression, and striatum-mediated alcohol consumption.

Female control mice exhibited habitual alcohol drinking

To our knowledge, this is the first evidence of LHb/*Bmal1*-mediated sex differences in alcohol consumption. However, LHb-dependent sexual dimorphisms have been found in various neural and behavioural functions. For example, there are sex differences in the inhibitory influence of the LHb on the midbrain DA neurons (Bell et al., 2023), and in LHb influence on social communication (Rigney et al., 2020), parental behaviour (Lecca et al., 2014), and stress responses (Zhang et al., 2018). As such, the LHb functions in a sexual dimorphic manner.

Here, female control mice retained high alcohol consumption throughout all paradigms, including the addition of quinine and after forced abstinence. This indicates that lowering the incentive value of the reward (i.e. alcohol) did not affect intake in control females. In animal models, persistent drug taking despite aversion or changes in reward value is defined as habitual drinking, a model of addiction-like behaviour (Dickinson, 1985; Vena et al., 2020). This shift to habitual behaviour is associated with a change from ventral (nucleus accumbens; NAc) to dorsal striatal control of drug taking (Balleine & O'Doherty, 2010; G. Chen et al., 2011; Graybiel & Grafton, 2015; K. S. Smith & Graybiel, 2016). This indicates that in females, *Bmal1* in the LHb may act by suppressing the shift from ventral to dorsal striatal control, inhibiting the shift to addiction-like drinking. While KO males demonstrated increased intake and preference after repeated alcohol exposure, they were significantly deterred by a change in the reward value (quinine and forced abstinence). As such, males do not meet the criteria for addiction-like alcohol drinking. Thus, we propose that *Bmal1* in the LHb of male mice may typically supresses

NAc related alcohol drinking behaviour but does not impact addictive-like drinking.

Notably, the difference in KO and control females was increased after the addition of quinine and after forced abstinence, the aversive paradigms. As the LHb plays an important role in aversive conditioning and acts as the so-called "negative reward center", this difference is likely due to a sex-specific role of *Bmal1* in mediating LHb functioning (Fu et al., 2017; Mondoloni et al., 2022; Zuo et al., 2014). The LHb typically has a weaker inhibitory influence on midbrain DA functioning in females (Bell et al., 2023) and estrogen in the LHb facilitates the response to stressful stimuli (Calvigioni et al., 2023). *Bmal1* may mediate LHb functioning through its control over estrogen. *Bmal1* binds to the E-Box in the promoter region of estrogen receptor β, controlling rhythmic oscillations (Cai et al., 2008). As estrogen receptors are denser in the female LHb (Bell et al., 2023), *Bmal1* may alter LHb functioning in a sex-dependent manner, thereby contributing to the observed sexually dimorphic drinking behaviour.

D2 receptors play a role in increased alcohol drinking behaviours

Sex differences in DA functioning have been well-established, especially in the striatum (Becker, 1990; Calipari et al., 2017; Dewing et al., 2006; McArthur et al., 2007; Walker et al., 2006; Zachry et al., 2021). Previously, we found that deleting *Bmal1* in the LHb indirectly alters striatal DA functioning (Goldfarb et al., *in prep*). Striatal DA is essential for clock gene functioning (Hood et al., 2010; Imbesi et al., 2009) and striatal clock genes, including *Bmal1* and *Per2* impact alcohol drinking behaviour in a sexually dimorphic manner (de Zavalia et al., 2021). As such, the sex- dependent drinking patterns found here may in part be due to LHb *Bmal1* mediated changes in DAergic functioning.

Moreover, the shift from ventral to dorsal striatal control is also associated with changes in balance between the direct and indirect striatal pathways. DA in the striatum functions primarily through these two pathways, D1 receptors mediate the direct pathway and D2 receptors mediate the indirect pathway. The interaction between DA and clock genes in the striatum is also cell type specific, as D1 and D2 receptor agonists influence clock gene functioning differently (Hood et al., 2010; Imbesi et al., 2009). Moreover, stimulation of D1 and D2 receptors differentially impact alcohol drinking behaviour, emphasizing the distinct role of these circuits (Cheng et al., 2017). Therefore, the impact of LHb *Bmal1* on alcohol drinking behaviour may be through cell type specific DAergic mechanisms in the striatum.

For example, D2 receptors on striatal medium spiny neurons (MSNs) have been linked to alcohol use disorder in humans (Hietala et al., 1994; Tupala et al., 2001; Volkow et al., 2019). Likewise, chronic alcohol consumption in mice reduced D2 receptor availability (Feltmann et al., 2018), and conversely, increase in the availability of D2 receptors in rats to reduced alcohol preference and intake (Thanos et al., 2001). After selective deletion of D2 receptors on MSNs, both male and female mice increased alcohol preference (Bocarsly et al., 2019). This is theorized to be due to the subsequent imbalance between the direct and indirect (D1 and D2) pathways, therefore resulting in altered striatal circuitry and the potential for increased vulnerability to alcohol. The present findings demonstrate that *Bmal1* in the LHb typically decreases the propensity for alcohol drinking in males and increases it in females. This, along with our previous findings that LHb *Bmal1* influences DA functioning, allows us to speculate that *Bmal1* in the LHb may impact alcohol drinking behaviour by indirectly altering the balance of striatal DA circuitry.

The complexity of these interactions highlights the need for a more comprehensive understanding of the molecular and neural circuit mechanisms underlying the sex-specific regulation of alcohol consumption by *Bmal1*. These insights are essential for developing targeted therapeutic strategies for alcohol use disorders, which often exhibit sex differences in prevalence and severity. By elucidating the specific pathways and molecular players involved, future research can pave the way for more effective treatment.

Method

Subjects

Bmal1 floxed mice (B6.129S4(Cg)-Arntl^{tm1Weit}/J) were originally obtained from Jackson Laboratories. 43 mice were used in total, 19 males and 24 females. Animals were divided into two groups, A and B. Group A consisted of 11 males (6 control/ 5 knockout) and 15 females (8 control/ 7 knockout). Group B consisted of 8 males (4 control/ 4 knockout) and 9 females (4 control/ 5 knockout).

Male and Female mice were group-housed under a 12:12 light-dark (LD) cycle at 21 ± 2°C and 60% relative humidity with food and water available *ad libitum* prior to the stereotactic delivery of viral vectors at 12 – 16 weeks of age. The bedding of the cages was changed every week. Animals were housed individually after the surgery under the same housing conditions as

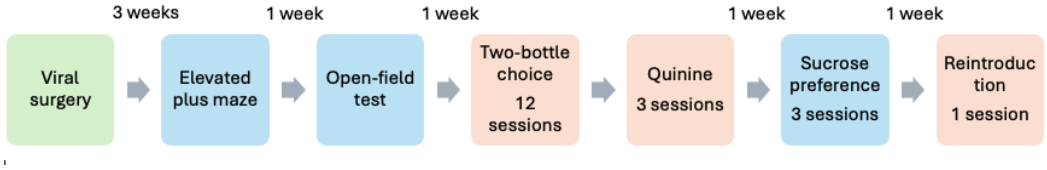
described above.

All experiments were conducted according to the guidelines and requirements of the Canadian Council on Animal Care (CCAC) and approved by the Concordia University ethics committee (AREC number 3000256).

Stereotaxic Surgeries

Bmal1 floxed mice were anesthetized by intraperitoneal injection of Ketamine (100 mg/kg body weight) and Xylazine (10 mg/kg body weight) solution and received subcutaneous injections of Ketoprofen (5 mg/kg body weight) as post-operational analgesia. Animals were placed in a stereotaxic apparatus (KOPF, Tujunga, CA, USA) and received bilateral intra-LHb microinjections of recombinant viruses (AP: -1.65, ML: 0.4, and DV: -3.1) using a 30-gauge needle attached to a 10 ul Hamilton syringe connected to a micropump (Harvard Aparatus). The injector was inserted at a 0° angle and the virus was delivered at a rate of 100 nl/min (120 nl total volume). For the generation of conditional *Bmal1* knockout, mice were given recombinant viral vectors expressing Cre and enhanced green fluorescence protein (eGFP) (AAV2/9-CAG-Cre-eGFP, $1x10^{12}$ vg/ml). Control animals received intra-LHb viruses expressing eGFP only (AAV2/9-CAG-eGFP, $1x10^{12}$ vg/ml). The injector was left in place for five minutes following the injection to optimize diffusion. Mice were allowed to recover for three weeks before behavioural testing. LHb-specific expression of viral vectors was evaluated at the end of the experiment. Animals with missing, incomplete, or off-region eGFP expression were excluded from all experiments.

Behavioural procedures, Ethanol consumption and Sucrose preference



Group A (11 males (5 KO); 15 females (7 KO) were assessed on the elevated plus maze (EPM), open-field test (OFT), two-bottle choice, quinine, sucrose preference test, and reintroduction session. Animals were euthanized after the reintroduction session and brains were collected for viral validation.

Alcohol naïve group B (8 males (4 KO) 9 females (5 KO)) was assessed only on the drinking in the dark paradigm. Animals were euthanized after the fourth session and brains were collected for viral validation.

Elevated Plus Maze

The elevated plus maze was used as an assay for anxiety-like behaviours. The "+"-shaped maze was positioned 40 cm above the ground with two enclosed arms (6 x 29.5 cm), two open arms (6 x 29.5cm) and a center area (6 x 6 cm). White methacrylate floors and black walls (15 cm in height) lined the bottom and enclosed arms of the maze, respectively. The test is based on mice's natural aversion to heights and open areas (Walf and Frye, 2007). Mice were habituated to the testing room by leaving them in their cage for an hour before the start of the experiment. Then, each mouse was placed in the center area of the elevated plus maze (Harvard Apparatus, Holliston, MA, USA) facing the closed arm and allowed to explore the maze for 5 minutes while video recorded (Samsung Galaxy A5 2017 phone). Video files were analyzed as described in Schoettner et al. (2022). Time spent in the open arms and number of entries into the open arms were assessed. The test was conducted at Zeitgeber time 2 (ZT2, ZT0 represents the time of lights-on).

Open field test

The open field test was used to assess anxiety-like behaviour and motor activity. After a 1-hour habituation period in the experimental room, mice were placed in the corner of the open field arena (45 x 45 x 60 cm, Panlab, Barcelona, Spain) facing the wall. The open field was equipped with infrared beams to track horizontal activity over 30 minutes using the ACTItrack software (Panlab, Barcelona, Spain). At the end of the session, animals were weighed and returned to home cages while the arena was wiped with a 70% ethanol solution (v/v in tap water) to remove any residues and olfactory cues before the next set of animals were tested. Total distance travelled, permanence time in the center of the open field, and the latency to enter the center were assessed. The test was conducted at ZT 2.

Intermittent alcohol exposure two-bottle choice

Voluntary alcohol consumption was studied using the intermittent exposure two-bottle choice test (IAE) as described previously (de Zavalia et al., 2021). Briefly, mice were habituated to drinking water from two bottles for three days, one week after the last behavioural test. They were then given access to one bottle of alcohol solution (15% v/v, in tap water) and one bottle containing tap water every other day, for 12 sessions. The position of the alcohol and water bottles was altered each session to control for potential side preference. All mice were given access to two water bottles during the alcohol-off days. Alcohol and water intake (g/kg body weight/day) and alcohol preference (ml of alcohol solution consumed/total fluid intake) were measured daily and calculated at the end of the 12 sessions. Body weights were collected weekly during cage changes.

Quinine and alcohol

Continuous drinking despite aversive consequences is a DSM-5 diagnostic criteria for alcohol use disorder. One way this is modeled in animal voluntary drinking paradigms is through the addition of quinine to the alcohol solution. Quinine is a bitter tasting substance and its addition to ethanol alters the valence of alcohol, making the solution less palatable. The same procedure as described above for two bottle choice was used. In brief, mice were given one bottle of 15% ethanol containing 250 uM quinine in tap water (v/v) and one water bottle for three consecutive days. Bottle placement was swapped every day. Alcohol and water intake (g/kg body weight/day) were measured daily and calculated at the end of the three sessions. Body weights were collected in morning before the first session.

Sucrose preference

A sucrose preference test was performed one week following the alcohol/quinine test. To minimize the effects of neophobia as a confounding factor, animals were given two bottles of 1% sucrose solution (v/v in tap water) for 4 hours the day before testing, and the volume of consumed sucrose solution was assessed to ensure all animals were drinking. During the test, mice were given unlimited access for 24-hours to one bottle of tap water and one bottle of 1% sucrose solution for three consecutive days, with the position of the sucrose and water bottles switched every day to control for side preference. The ratio of the sucrose solution consumed

relative to the total fluid intake was used as a measure of sucrose preference ($V_{sucrose\ solution}/(V_{sucrose\ solution} + V_{water})$).

Reintroduction

After a period of forced abstinence (1 week after SPT; 10 days after the completion of the 3-day quinine/ alcohol paradigm) mice were reintroduced to one bottle of 15% alcohol in tap water (v/v) and one bottle of tap water for a single 24-hour session to investigate the effect of LHb *Bmal1* deletion on relapse-like drinking. Alcohol and water intake (g/kg) were measured and calculated at the end of the session. Body weights were collected after the session.

Drinking in the dark test

To assess binge drinking behaviour, alcohol naïve mice underwent a four-day drinking in the dark paradigm (Thiele et al., 2014). In brief, mice were given 2h access to one bottle of 20% ethanol in tap water (v/v) for 3 consecutive days, starting 2 hours into the dark phase (ZT14), and for 4 hours on the fourth day. Body weights were measured before the first session and at the end of the last session. Alcohol intake (g/kg body weight/day) was measured each day and calculated at the end of the final session.

Immunofluorescence

Animals were deeply anesthetized by exposure to an atmosphere of isoflurane and perfused transcardially by cold saline (0.9% sodium chloride, pH 7.2) followed by paraformaldehyde solution (PFA, 4% in 0.1M phosphate buffer, pH 7.2) using an infusion pump. Brains were then dissected and postfixed in PFA for 22 – 24h at 4°C. 30 µm coronal section of brain tissue were collected using a Leica vibratome and analyzed under a fluorescent microscope to validate region-specific viral vector delivery. Brain slices were stored at −20 °C in Watson's cryoprotectant (Watson et al., 1986) for immunofluorescence imaging.

For this, free-floating sections previously kept in Watson's cryoprotectant were rinsed for 10 minutes in phosphate buffered saline (PBS, pH 7.4). Following this, sections were washed three times in PBS containing 0.3 % Triton-X (PBS-Tx) for 10 min, followed by an incubation in blocking solution (3 % milk powder, 6 % normal donkey serum (NDS) in PBS-Tx) for one hour at room temperature with mild agitation. Primary antibody solution was prepared by diluting the

primary antibody for BMAL1 (rabbit anti-BMAL1, 1:500, NB100-2288, Novus Biologicals) in PBS-Tx containing 3 % milk powder and 2 % NDS. Brain sections were incubated for 1.5 hours at room temperature. Following three washes in PBS-Tx, brain sections were incubated at room temperature in secondary antibody solution containing donkey anti-rabbit IgG Alexa Fluor 647 (1:500, Thermo Scientific^{TM})) diluted in PBS-TX (3 % milk powder and 2 % NDS) for one hour. Brain sections were mounted on gel coated microscope slides and cover slipped using a mounting media containing DAPI (ProLong™ Diamond Antifade Mountant, Thermo ScientificTM) after three washes 10-min in PBS-Tx and a final wash in PBS for 10 min. Brain sections were stored at 4 °C until imaging under an Olympus 10FI confocal microscope. A ratio between BMAL1 expressing cells over GFP expressing stained cells was used to calculate rate of viral infection. Representative images were prepared using Image J.

Data analysis and statistics

The collected raw data from the behavioural tests and alcohol consumption experiments were further processed and analyzed using Prism 9 (GraphPad Software, San Diego, CA, USA). Unpaired two-tailed t tests and repeated measures ANOVAs were used to compare differences between groups. Significant main effects and interactions of the ANOVAs were further investigated by the appropriate post-hoc test. The level of statistical significance was set at $P < 0.05$. Exact P values and further details are given in the tables above. The outcomes were depicted as mean ± standard error of the mean (SEM).

Author contributions

K.S., S.A., and C.G. conceived and designed the study; C.G., V.H., and A.M. performed the experiments; C.G., K.S., and S.A. analyzed and interpreted the data, and wrote the manuscript; S.A. and K.S. Supervision.

Competing interests

The authors declare no competing interests.

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General Discussion

Dopamine (DA) is essential for a number of behaviours, including motor functioning and reward. However, it is not only DA's *presence* that is essential, but also its *rhythms*. Previous research has established that rhythmic activity of DA projections into the striatum is vital for regulating the striatal clock (Hood et al., 2010; Imbesi et al., 2009) and this clock is essential for typical motor functioning (Schoettner et al., 2022) and alcohol drinking behaviour (de Zevalia et al., 2021). The question that remained, and the purpose of the present thesis is: what controls DA's rhythm? We hypothesized that the habenula (LHb) is acting as a pacemaker for rhythmic DA synthesis and release. We also explored what the impact would be on DA related behaviours after disrupting this pacemaker.

To test our hypotheses, we virally knocked out *Bmal1* in the LHb of male and female mice and assessed them for behavioural and molecular functioning. We found significantly attenuated motor functioning and attempted to rescue these deficits using both pharmacological and non-pharmacological approaches. Additionally, we examined the effect of *Bmal1* deletion in the LHb on alcohol drinking behaviours, finding sexually dimorphic drinking patterns. These results highlight the importance of the LHb clock in regulating striatal dopamine-related behaviours, supporting our hypothesis.

LHb *Bmal1* KO resulted in impaired motor functioning

Our findings provide compelling evidence that the LHb plays a role in regulating the rhythmic functioning of DA. Targeted knockout of *Bmal1* in the LHb significantly impacted motor functioning, DA levels, and gene expression in the substantia nigra (SN) and dorsal striatum (DS). Analyses revealed altered rhythms in clock genes and DAergic-related genes in SN of *Bmal1* knockout mice. This supports previous findings that clock genes in the SN influence the rhythmicity of DAergic synthesizing enzymes, such as *Th* and *Maoa* (Chung et al., 2014; Mcclung et al., 2005). Chung and colleagues found a direct effect of *Rev-erb* on *TH* in the SN, where *Rev-erb* supresses *TH* in a circadian manner, with *Rev-erb* peaking in the day and *TH* peaking in the night (2014). *Rev-erb* KO mice therefore lose this rhythmicity in *TH* expression, and levels are elevated throughout the day. Similarly, we found that *Rev-erb* was elevated throughout the day in LHb *Bmal1* KO mice, resulting in blunted levels of SN *TH*. Additionally, rhythms of striatal DA were blunted in KO mice, similar to those found after a *Per2* KO blunted

Maoa levels and altered striatal DA (McClung et al., 2005). Moreover, previous findings indicate that a global *Bmal1* KO leads to the loss of TH+ neurons in the SN (Kanan et al., 2024; Liu et al., 2020) highlighting the need for a functional clock for typical DA signalling. Thus, it is likely that DA levels in the striatum were altered in our sample due to changes found in SN clock functioning.

This study provides a comprehensive investigation into the interplay between circadian rhythms and DA regulation, with significant implications for understanding how the LHb's clock moderates this relationship. This study also highlights that disruptions to the rhythmic signals for DA's production can negatively influence associated behaviours.

Daily therapeutic strategies can partially restore motor functioning

Building on these findings, our second chapter aimed to rescue the motor deficits observed previously in mice with LHb-specific *Bmal1* KO. These mice exhibited impaired performance in motor behaviours, altered SN gene expression, and changes in daily striatal DA levels. Due to these findings, we concluded that *Bmal*1in the LHb is essential for rhythmic functioning of DA neurons that project from the SN to the DS.

To restore motor functioning, we used pharmacological (quinpirole) and non-pharmacological (running wheels) interventions. Quinpirole, a D2 agonist, was used to restore striatal rhythms, as previous research indicated its effectiveness in restoring PER2 rhythms in rats with blunted rhythms resulting from 6-hydroxydopamine (6-OHDA) lesions of the medial forebrain bundle (Hood et al., 2010). Moreover, running wheels were used as a non-pharmacological intervention due to ample evidence demonstrating that running wheel use can entrain circadian rhythms (Edgar et al., 1991; Edgar & Dement, 1991; Yamanaka et al., 2013).

Both interventions improved pole test performance, but quinpirole injections resulted in limited improvement in rotarod performance which was statistically significant only in females. Similarly, after pharmacological intervention, we found sex differences in SN levels of *Bmal1* and *TH*. These findings suggest that females were more strongly impacted by daily quinpirole injections, resulting in their improved motor functioning on both tests.

Despite globally improved motor function in the non-pharmacological intervention group, DA levels were not entirely normalized, suggesting that alterations to the DA system is not alone in causing the observed motor phenotypes in *Bmal1* LHb KO mice.

In conclusion, this study found that both pharmacological and non-pharmacological interventions can mitigate motor deficits in LHb-specific *Bmal1* knockout mice. The differential effects of these interventions on the pole test and rotarod highlight the complexity of motor functioning and the distinct neural mechanisms underlying each behaviour. Future studies should explore the potential combinational effects of these interventions to develop more comprehensive strategies for treating motor disorders linked to circadian disruptions.

Male Mice Drank More After Lhb *Bmal1* KO

Our third study identifies the LHb as a novel site for the sexually dimorphic effects of *Bmal1* on alcohol consumption. In male mice, *Bmal1* deletion in the LHb led to increased voluntary alcohol intake in the two-bottle choice test and amplified binge-like drinking behaviour. In females, a LHb *Bmal1* KO suppressed drinking of the bitter alcohol solution and reintroduction drinking levels following a period of forced abstinence. These results suggest that *Bmal1*-dependent mechanisms in the LHb typically repress alcohol consumption in males and facilitate consumption under aversive (quinine) or stressful (forced abstinence) conditions in females.

We speculated that the sex-dependent effects of *Bmal1* in the LHb on alcohol drinking behaviour may be related to the shift from ventral to dorsal striatal control found in addictive-like samples. Moreover, imbalances in striatal circuitry, specifically potentiation of increased D1 direct pathway functioning was previously found to result in increased alcohol preference (Bocarsly et al., 2019). As such, we theorized that LHb *Bmal1* may play a role in the maintaining the balance between the two pathways.

This study underscores the complexity of interactions between the LHb, striatum, and DAergic circuits in regulating alcohol drinking behaviour. Understanding these sex-specific regulations can inform targeted therapeutic strategies for alcohol use disorders.

Sex as a Biological Variable

Historically, biological research has primarily used male subjects, often excluding females due to concerns over hormonal fluctuations and variability (Beery & Zucker, 2011; Kubota et al., 2016). This is especially apparent in chronobiology research, where 81.7% of studies from 1964-2017 used only males (Lee et al., 2021). However, recent policies have been

put in place calling for the inclusion of both sexes in research (CIHR, 2019). In line with this, the present study utilized both male and female mice, revealing significant sex differences in responses to daily quinpirole injections and alcohol drinking behaviour.

Sex differences in LHb functioning may contribute to these observed outcomes. The LHb appears to exert a stronger inhibitory influence on midbrain DA neurons in males than in females, possibly due to the higher density of estrogen receptors in the female LHb, which have been shown to affect LHb functioning through calcium channel regulation (Bell et al., 2023; Li et al., 2015; Song et al., 2018). Differences in LHb functioning have been found in various behaviours, including stress related behaviours (Zhang et al., 2018), social behaviours (Rigney et al., 2020), and parental behaviours (Lecca et al., 2023). This differential modulation by estrogen could affect the balance between excitatory and inhibitory inputs, thereby influencing downstream DAergic targets.

DA itself exhibits sex-specific regulatory mechanisms. In females, DA release is more sensitive to stimuli, and feedback inhibition is reduced, leading to higher responding to DAergic stimuli under certain conditions (Becker & Chartoff, 2019; Walker et al., 2006). Estrogen further enhances these effects, as higher estrogen levels have been associated with increased alcohol consumption in females, linked to interactions with the DAergic system (Barth et al., 2015; Prendergast et al., 2014). Higher levels of DA neurons in the male SN and in the female VTA also lead to increased sex- and pathway- dependent DA effects (Dewing et al., 2006; McArthur et al., 2007). Thus overall, there is ample evidence of sex differences in DAergic functioning.

The circadian system also interacts with sex hormones, influencing behaviours and physiological functions in a sex-dependent manner. Circadian disruption affects estrus cycling (Fahrenkrug et al., 2006; Sellix, 2015) and sex hormones have been found to act directly and indirectly on the central SCN clock (Pinto & Golombek, 1999; Trachsel et al., 1996). Free-running period length is typically shorter in females, and ovariectomy results in an increase similar to males (Albers et al., 1981). Female mice normally have a stronger phase shift reaction to a light pulse at night (Kuljis et al., 2013), and this response appears to be estrogen mediated (Blattner & Mahoney, 2012). Similar results are found for testosterone, as male gonadectomized mice demonstrate larger phase shifts than intact males (Karatsoreos et al., 2011). Moreover, some striatal clock genes and clock-controlled genes demonstrate sex-dependent rhythms (Schoettner et al., 2022), and women have more rhythmic genes than men do (Talamanca et al.,

2023). These findings suggest that sex hormones as well as biological sex both play a role in mediating sexually dimorphic circadian behaviours.

In summary, the sex differences observed in the present thesis may be due to complex interactions between sex-specific factors and the LHb, DA regulation, and the circadian system. This highlights the importance of studying sex as a biological variable and need to for research to continue including female samples.

The Habenula as Dopamine's Pacemaker

Current research on the brain mechanisms controlling specific behaviours and physiological processes is shifting. Rather than distinct brain regions or neuronal populations being solely responsible, entire networks working in tandem are regulating the phenotypes we see. Critically, these networks, or components of them, undergo tremendous diurnal alterations in their function that contribute, directly or indirectly, to alterations in behaviour. Thus, while this thesis focused on clocks within a small number of regions associated with DA functioning, it must be recognized that this is only a piece of a complex network. The primary focus of this thesis was on the peripheral clocks in the LHb, SN, and DS, and in particular on the influence of the clock in the LHb's on DAergic system and several DA-dependent behavioural and molecular processes.

While the LHb clock clearly plays a strong role in regulating rhythmic DA, neither daily quinpirole injections nor running wheels fully rescued clock gene and DA levels, despite improvement in motor functioning. This suggests, that while DA is an integral part of these behaviours, it functions as part of a larger, more complex system.

Key to LHb functioning is not only its projections to the midbrain DA nuclei, but also its projections to the dorsal raphe (DR) – the major center for serotonin (5-HT) production. Most research involving the LHb and its efferent pathways has focused on its role in DA regulation, but the LHb also shares strong connections with the DR. Similar to its control over DA, LHb signals suppresses 5-HT release from the DR (Metzger et al., 2017). In turn, 5-HT receptors also regulate DAergic activity (Alex & Pehek, 2007), resulting in a strong influences of 5-HT in regions such as the VTA, SN, and striatum (Ogawa et al., 2014; Vertes, 1991). In rats, 6-OHDA lesions, to the medial forebrain bundle, resulted in increased LHb activity and depressive-like behaviours. When the LHb was also lesioned, 5-HT levels in the DR were increased and

depressive-like behaviours were reduced (Luo et al., 2015). Furthermore, the enzyme converting tryptophan to 5-HT also converts L-DOPA to DA, facilitating a role for L-DOPA in mediating DAergic functioning (Carta et al., 2007). In PD, decreased DA release after SN cell death is initially compensated by lower reuptake transporter function, leading to increased extracellular DA. However, mathematical modelling has found that after 80% cell death of SN DAergic neurons, this homeostatic mechanism fails. In this situation, striatal 5-HT is increased which results in more DA released, temporarily compensating for the loss (Reed et al., 2012, 2013). Thus, 5-HT plays a strong role in modulating DAergic functioning, especially in the striatum.

Additionally, there are also substantial connections from the DAergic system to the raphe nucleus (Ogawa et al., 2014). Specifically, D2 receptors have been found in the DR (Okaty et al., 2019) and D2 receptor agonist quinpirole has been found to moderate DR activity (Haj-Dahmane, 2001). This suggests that in our study, daily quinpirole injections would also have impacted DR functioning and in turn 5-HT along with D2 activation.

Furthermore, 5-HT affects striatal functioning through its effect on striatal cholinergic interneurons. Cholinergic interneurons in the nucleus accumbens (NAc) mediate reward and motivation, while in the dorsal striatum (DS), they are implicated in motor functioning and habitual behaviour (Apicella, 2002; Virk et al., 2016; Warner-Schmidt et al., 2012; Witten et al., 2010). 5-HT inhibits NAc cholinergic interneurons but excites those in the DS. Loss of serotonin receptor 5-HT1b in the NAc leads to increased anhedonia, but does not appear to affect DS motor functioning (Virk et al., 2016). Conversely, 5-HT1A agonists can mediate some motor symptoms in PD by activating cholinergic receptors in the DS (Jiang et al., 2022), demonstrating 5-HT's role in mediating DS functioning through cholinergic interneurons.

The intricate interplay between the LHb, DA, and 5-HT underscores the complexity of behavioural regulation and likely underlies many of the behaviours investigated in this thesis. While the primary focus of this thesis was on the LHb's influence over the functioning of the DAergic system and the behavioural and molecular alterations associated with ablation of the LHb clock, it is likely that other neuronal system influence these processes. Future research should expand to directly assess the LHb’s clock impact on the 5-HT system to further elucidate these complex interactions and their implications.

Dopamine as a Zeitgeber

Ample evidence has indicated a strong bi-directional relationship between the DAergic system and the circadian system. This supports the theory that dopamine acts as a zeitgeber, setting the pace for clocks such as the striatal clock. Normal DAergic functioning is necessary for typical activity rhythms; for example, a D2 knock-in led to lower day/night activity variations (Doi et al., 2006). Previously, this effect was attributed to the influence of retinal DA levels, but blunted *Per2*, *Dbp*, and *Bmal*1 levels in the striatum highlighted its role in daily activity rhythms (Sahar et al., 2010). Further, Hood and colleagues found that striatal *Per2* rhythms were abolished after 6-OHDA lesions of the medial forebrain bundle (2010). Thus, rhythmic DA is essential for striatal clock functioning, and its loss can lead to impaired motor functioning (Hood et al., 2010).

This effect extends beyond the striatum and nigrostriatal pathway. Clock genes in the VTA regulate DA synthesis and release, leading to rhythms in reward (Hampp et al., 2008; Mcclung et al., 2005; Mukherjee et al., 2010; Roybal et al., 2007). For instance, rats given free access to cocaine consume more during the dark phase (active phase), likely due to high nighttime levels of DA and its effect on reward seeking. Consequently, DA's impact on the striatal clock could render it, and therefore the striatum, vulnerable to any changes in DAergic functioning, thereby leading to disorders such as PD or exacerbating addiction. Therefore, studies focusing on treatments targeting the DAergic system in these disorders must account not only for the presence of DA but also its timing. Thus, the strong relationship between DA and the circadian system can open novel avenues for therapeutic interventions. For example, in the present study, timed daily injections of quinpirole and access to running wheels mostly restored motor performance.

The largest area of research into circadian-based treatment interventions focuses on light therapy. Some studies have found that daily morning light therapy positively impacts motor functioning in Parkinson's disease (PD) (Artemenko & Levin, 1996; Paus et al., 2007; Willis & Turner, 2007), though more clinical trials are still needed as the methodology of some studies has been questioned (Huang et al., 2021; Lin et al., 2021). Melatonin supplements have been shown to improve some PD symptoms (Bolitho et al., 2014), especially when combined with typical L-DOPA treatment, which is more effective when taken in the morning (Li et al., 2017). Similarly, intermittent fasting (time-restricted eating) protected SN DA in a PD mouse model and improved locomotor activity and striatal DA levels in primates (Maswood et al., 2004;

Matson & Grahame, 2013). While no clinical results have been presented yet, studies such as those presented above, suggest that targeting the circadian system in treating PD may be an effective tool. As such the limited evidence that circadian based interventions can benefit PD symptoms to some extent contribute to better understanding of these interconnected networks.

Circadian-based treatment interventions for addiction have also been assessed. Melatonin supplements have been shown to alleviate the negative symptoms of withdrawal and to reduce the addictive nature of some drugs (for review, Jia et al., 2022). Specifically, melatonin can reduce alcohol drinking bouts in free-access paradigms (Vengeliene et al., 2008). As well, Diazepam, a common treatment for alcohol withdrawal, is more effective and led to higher absorption rates when given in the morning (Nakano et al., 1984). As such, the circadian system is an important target for addiction treatment.

Importantly, knowing that symptoms of PD and addiction vary by time of day, such as motor deficits or drug craving tend to worsen at night, circadian-based treatments targeting the DAergic system could then be implemented at the most effective time of day. Understanding the bidirectional relationship between the DAergic and circadian systems, as well as specific brain regions that mediate this relationship, will aid in finding new and more effective strategies for treating disorders such as PD and addiction.

Conclusions

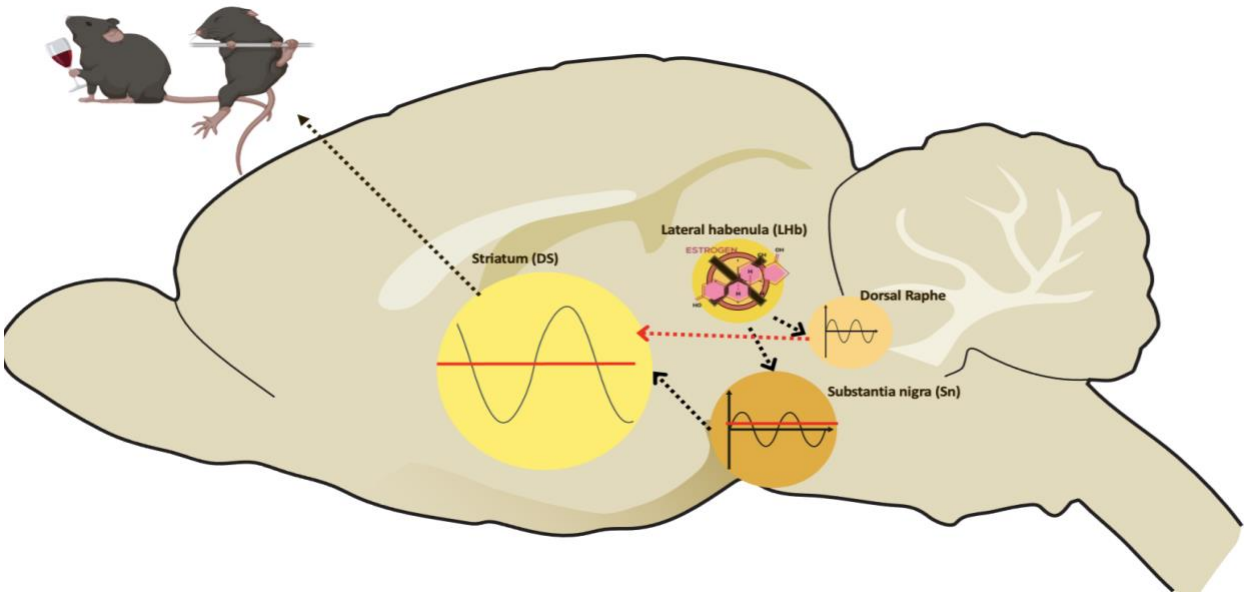

Figure 4-1 Proposed Circuit for the Rhythmic Control of Nigrostriatal Dopamine. Proposed circuit outlining the role of the LHb clock in moderating rhythmic dopamine release. Additional roles for serotonin and estrogens are noted.

In summary, this thesis has demonstrated the critical role of the LHb clock in regulating rhythmic DA release and its broader implications on motor functioning and alcohol drinking behaviours. The studies included in this thesis provide compelling evidence that the LHb acts as a pacemaker for DA rhythms, and that these rhythms are essential for motor functioning. Furthermore, this thesis identifies *Bmal1* in the LHb to have a sexually dimorphic effect on alcohol drinking behaviour, with only male mice demonstrating increased alcohol intake after a LHb specific *Bmal1* knockout. These results underscore the intricate relationship between the LHb, DAergic system, and sex hormones in regulating alcohol drinking behaviour and support DA's role as a zeitgeber in setting the pace for the striatal clock. The bidirectional relationship between the DAergic and circadian systems offers novel insight into additional therapeutic avenues for DAergic disorders.

In conclusion, this thesis advances our understanding of the LHb's role in regulating DA rhythms and the resulting behavioural and molecular alterations. The findings pave the way for future research into therapeutic strategies that account for the complex interactions between circadian clocks and the DAergic system, with attention to sex-specific responses. Understanding these intricate networks will aid in our understanding of neuronal functioning and support the development of more effective treatments for disorders related to both the DAergic and circadian systems.

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