

Discovery of a novel rhythm generation process in the mammalian brain guided by lessons from the circadian timing system

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Abstract:

The master circadian pacemaker within the suprachiasmatic nucleus entrains to daily cycles of light and darkness, conveying this information throughout the brain and body to modulate physiological and behavioural processes in synchrony with the external environment. It achieves sensitivity to incoming cues, precision, robustness, and coordinated output via neural network mediated mechanisms which we hypothesize to rely on specific subpopulations of neurons delineated by neuropeptide expression. By selectively ablating or resurrecting circadian molecular clocks within one of these subpopulations, expressing vasoactive intestinal peptide, we hoped to impinge upon one specific node in this network and discover some of the network properties of this nucleus. Indeed, our results demonstrate that clocks within VIP expressing cells are necessary, but not sufficient, for appropriately timed behavioural rhythmicity and responses to light. Furthermore we have identified this subpopulation as intrinsic pacemaking neurons capable of self-synchrony in the absence of all exogenous timing cues. We can also rule out VIP-expressing cells as the drivers for the expression of locomotor output.

It is distinctly possible that certain processes must occur more frequently than once per day. Our observation of behavioural oscillations that typically cycle 6 times in a single 24 hour period suggests that the mammalian brain has evolved mechanisms to anticipate and/or drive such events. We hypothesized, based on our behavioural observations in circadian compromised mice, that these oscillations relied upon a distinct biological clock, possibly residing in the brain of mammals. We demonstrated the existence of this neural system, which we have dubbed the dopaminergic ultradian oscillator (DUO), using pharmacological, genetic, and network-restricted chemogenetic tools. Our data proves that the DUO resides specifically within dopaminergic cells themselves, thereby providing us with the opportunity to use genetically targeted strategies for dissecting its neuroanatomy and function while simultaneously identifying and modelling various disease states which might occur if this system goes awry.

Résumé:

L'oscillateur circadien principal, situé dans le noyau suprachiasmatique (NSC), s'ajuste aux cycles jour-nuit et communique cette information à travers le cerveau et le corps, afin de moduler les processus physiologiques et comportementaux selon les rythmes environnementaux. La sensibilité aux signaux entrants, la précision et la robustesse de l'oscillateur, et les voies de sortie de celui-ci reposent sur un réseau de neurones dans le NSC. Notre hypothèse était que ce réseau est rendu possible grâce à des sous-populations spécifiques de neurones délimitées par l'expression du neuropeptide. Par ablation ou résurrection sélective d'horloges circadiennes moléculaires dans l'une de ces sous-populations, exprimant le peptide intestinal vasoactif (VIP), nous pensions affecter un élément central de ce réseau et ainsi découvrir certaines de ses propriétés du réseau de ce noyau. De fait, nos résultats démontrent que les horloges dans les cellules exprimant VIP sont nécessaires mais pas suffisantes pour la rythmicité comportementale. En outre, nous avons démontré que cette sous-population de neurones est capable d'auto-synchronisation en l'absence de toute indication temporelle exogène. Nous pouvons également exclure les cellules exprimant VIP de la liste des populations cellulaires initiant l'activité locomotrice.

Certains processus doivent par ailleurs se produire plus fréquemment qu'une fois par jour. Notre observation de rythmes comportementaux qui se produisent 6 fois en une seule période de 24 heures suggère que le cerveau des mammifères a développé des mécanismes pour anticiper ou initier de tels événements. Nous avons émis l'hypothèse, sur la base de nos observations comportementales chez des souris dont les rythmes circadiens sont compromis, que ces oscillations sont le fruit d'une horloge biologique distincte, possiblement située dans le cerveau des mammifères. À l'aide d'outils pharmacologiques, génétiques et chimiogénétiques spécifiques, nous avons démontré l'existence de ce système neuronal, que nous avons baptisé l'oscillateur ultradien dopaminergique (DUO). Nous avons démontré que le DUO réside spécifiquement dans les cellules dopaminergiques elles-mêmes, nous offrant ainsi la possibilité d'utiliser des stratégies ciblées génétiquement pour disséquer sa neuroanatomie et sa fonction tout en identifiant et en modélisant divers états pathologiques qui pourraient survenir si ce système se dérègle.

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I would like to thank my supervisor, Dr. Florian Storch, for the exemplary lead. For showing me that risk is well worth the reward and that discovery is not as daunting as it may sometimes appear but that it does take grit, determination and a pioneering spirit.

To the other professors who have supported my growth as a scientist and my research at the Douglas, Dr. Cermakian, Dr. Giros, Dr. Gratton, and Dr. Kokoeva, I thank you for your support.

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I would like to thank my parents for their unwavering support all of these years, through the long and winding path. Mom, Dad, this research is dedicated to you.

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Preface:

Contributions of authors:

As the works contained in this thesis were collaborative in nature I would be remiss if I did not explicitly state the contribution of each participant. Dr. Lei Zhu was integral to both the brain tissue collection and data analysis of the HPLC data in *Bmal1*^{-/-} mice after methamphetamine treatment (Manuscript 2) he was also integral to the mouse colony maintenance and data collection for all running wheel experiments, helping me out whenever I was not able due to time or other work. Dr. Maia Kokoeva provided the use of all equipment, space, animals, and reagents necessary for all telemetric experiments throughout the entire thesis (Manuscript 2 and 3) as well as specific help in discussing and writing the original DUO Manuscript (Manuscript 2). Luc Moquin performed all HPLC (Manuscript 2). Dr. Alain Gratton provided all equipment, reagents, and considerable expertise in designing the experiments for all monoamine (and metabolite) quantification including the time of Luc Moquin, his research technician (Manuscript 2). Dr. Gregory Dal-Bo performed the iodosulpride binding assay quantification and analysis (Manuscript 3). Dr. Elsa Isingrini provided the animals, the molecular/anatomical verification, and her considerable expertise in handling, for the SERT-Cre- and DBH-Cre-flx-VMAT2 mice (Manuscript 3). Dr. Bruno Giros was constantly consulted for his expertise in the dopaminergic system throughout our characterization and later exploration of the dopaminergic ultradian oscillator, he contributed to the discussion of DUO results and generously provided all of the animals in the DUO manuscripts with a monoaminergic targeted phenotype including colony maintenance and genotyping (DAT^{-/-}, DRD1^{-/-}, DRD2^{-/-}, DAT-Cre, SERT-Cre, DBH-Cre, DRD2^{flx/flx}, and VMAT2^{flx/flx}). Dr Florian Storch contributed considerable time, effort, and any resources not previously listed, included my own stipend, the salary of Dr. Lei Zhu his research associate, and the salary of Aude Villemain, his technician and the primary caretaker his breeding colony (all circadian clock phenotypes, Manuscript 1 and 2). He participated in every step of this thesis; engaging with me directly in experimental design, discussing appropriate analyses, and interpretation of the observed behaviour and physiology. This of course brings me to my own contributions, which include designing, performing, and analyzing – or in the case of experiments explicitly done by a collaborator, analyzing and presenting—all experiments contained within. This also includes the preparation of all manuscripts and figures. In one case, (Manuscript 1) I was called upon to design (based upon the work of Dr. Shin Yamazaki) and construct the apparatus for bioluminescent imaging with single cell resolution. Furthermore any and

all ideas, including experimental design, analysis and interpretation of data, presented within this thesis are the direct result of not inconsiderable discussions between myself and Dr. Storch.

Original scholarship:

Throughout this manuscript, many novel experimental and analytical findings have been made but in the interest of brevity we can summarize the original scholarship presented here into two distinct contributions. The first is the development of genetic, optical, and analytical tools allowing for the genetic dissection of functional networks within the master circadian pacemaker of the mammalian brain, the suprachiasmatic nucleus. Specifically, we address the contributions of vasoactive intestinal peptide expressing cells within this network; their role in the gating of photic input, rhythm generation, circadian pacemaking, and locomotor output. Our second major contribution is the discovery of a novel rhythm generation process in the mammalian brain, one that critically relies on oscillation of dopamine for both rhythm generation and its output controlling locomotor rhythms on the ultradian scale (~ 4 h). We have dubbed this the dopaminergic ultradian oscillator (DUO) and as well as characterizing its oscillatory properties, we have made a significant effort to identify molecular targets which contribute to the feedback loops which underlie such oscillations. Finally, we suggest the novel hypothesis that it may be dysfunction of this biological clock which underlies psychopathology, especially when considering the oscillatory (if sometimes chaotic) nature of both schizophrenia and bipolar disorder.

Abbreviations:

5-HT	Serotonin	Hal	Haloperidol
AAV	Adeno-associated virus	HPLC-EC	High pressure liquid chromatography with electrochemical detection
α MPT	α -Methyl- <i>p</i> -tyrosine	iCRE	Improved cre-recombinase enzyme
ACKO	AVP-Cre ^{Tg} <i>Bmal1</i> ^{flx/flx}	IRES	Internal ribosomal entry site
ANOVA	Analysis of variance	KO	Knockout, as in gene knockout
AP-1	Activator protein 1	LD	Light-dark cycle
AS	AVP-Cre ^{Tg} <i>Bmal1</i> ^{fls/fls}	MAPK	Mitogen Activated Kinases
ASD	Amplitude Spectral Density	MASCO	Methamphetamine sensitive circadian oscillator
AVP	Arginine Vasopressin	Meth	Methamphetamine
BH4	Tetrahydrobiopterin	NMS	Neuromedin S
BMAL1	Brain and muscle ARNT-like protein 1	NorE	Norepinephrine a.k.a. noradrenaline
BT	Body temperature	NPY	Neuropeptide Y
CaMPKII	Calmodulin-dependent protein kinases	PACAP	Pituitary adenylate cyclase-activating peptide
CCGS	Clock controlled genes	PER1,2 & 3	<i>Period</i> proteins 1, 2, and 3
CK1 ϵ	Casein Kinase 1 Epsilon	PKA,B & C	Protein kinases A, B, and C
cKO	Slc6a3-Cre ^{Tg} <i>Drd2</i> ^{flx/flx}	PRC	Phase response curve
CLC	Cardiotrophin-like cytokine	PRK2	Prokineticin 2
CREB	Cyclic-AMP responsive elements binding protein	PRKr2	Prokineticin receptor 2
CRE	CREB response elements	pSer40	Phosphate bound serine residue number 40 of the tyrosine hydroxylase enzyme
CRY1 & 2	<i>Cryptochrome</i> Proteins	PVN	Paraventricular nucleus
CT	Circadian time	RE α & β	<i>Rev-Erb</i> Proteins
CWT	Continuous wavelet transform	RFP	Red fluorescent protein
BRET	Bioluminescent resonance energy transfer	RHT	Retino-hypothalamic tract
DAB	3,3'-diaminobenzidine	ROR α , β , & γ	RAR-related orphan receptors
DAT	Dopamine transporter	RORE	Retinoic acid response elements
DD	Constant Darkness	SCN	Suprachiasmatic nucleus
DUO	Dopaminergic ultradian oscillator	SCNx	Electrolytic lesions of the SCN
DOPAC	3,4-dihydroxyphenylacetic acid	SERT	Serotonin reuptake transporter protein
DR	Dorsal raphe	Slc6a3	Dopamine transporter gene
DRD1-5	Dopamine receptors D1 through D5	Slc6a4	Serotonin transporter gene
DREADD	Designer receptors exclusively activated by designer drugs	SNc	Substantia nigra pars compacta
<i>F</i>	F-statistic from analysis of variance	sPVZ	Hypothalamic sub-paraventricular zone
FAA	Food anticipatory Activity	TdTomato	Tandem dimer mutant of RFP
FACS	Fluorescent auto cell sorting	TH	Tyrosine hydroxylase enzyme
FDR	False discovery rate analysis	TNF- α	Tumor necrosis factor alpha
FEO	Food entrainable oscillator	TTFL	Transcriptional/translational feedback loop
flx	Floxed copy of gene for excision	VCKO	VIP-IRES-Cre ^{+/+} <i>Bmal1</i> ^{flx/flx}
fls	Floxed-Stop copy of gene for resurrection	VIP	Vasoactive Intestinal Peptide
FRET	Fluorescent resonance energy transfer	VMAT	Vesicular monoamine transporter
GHT	Geniculo-hypothalamic tract	VS	VIP-IRES-Cre ^{+/wt} <i>Bmal1</i> ^{fls/fls}
GIRK	G-protein-activated inward rectifying potassium channel	VSb	VIP-IRES-Cre ^{+/wt} <i>Bmal1</i> ^{fls/fls} <i>Bmal1</i> -luc ^{Tg}
GRP	Gastrin releasing peptide	VT	- VIP-IRES-Cre ^{+/wt} TdTomato ^{fls/fls}
h3MDq	DREADD variant for Gq signalling		

Chapter 1: Introduction

The mammalian circadian system represents perhaps the most widely studied rhythm generator on this planet. Its influence is ubiquitous, its necessity almost obvious. For what other event in our environment is as predictable, and necessary for life, as the rising of the sun in the east and its setting in the west. The purpose of this system is well known and its influence, especially when perturbed, is now at the forefront of our field with relevance to transmeridian travel, shift-work, heart disease, cancer, and even infectious disease. However, the underlying neurological substrate is still very much an unsolved mystery. While the advances in the past couple of decades since the genetic revolution of the early nineties have elucidated the canonical clock genes, the cogs in the machinery that work at the most basic level in almost every cell of our bodies, we have yet to understand circadian regulation at the systems level. The first objective of this dissertation was to probe this system; to perturb the network interactions between individual molecular oscillators which compose the master circadian pacemaker in the suprachiasmatic nucleus (SCN), and to try and reverse engineer the clockwork to gain a better understanding of the evolution of such a precise timekeeper and its inputs, pacemaker properties, and behavioural and physiological outputs. We then demonstrated the efficacy of such a strategy for a key subpopulation of vasoactive intestinal polypeptide (VIP) expressing cells.

From this first objective came an intriguing observation, certainly one that had been made many times by our contemporaries but was often ignored: that the removal of the circadian system in mammals (and indeed in other organisms as we will discuss later) revealed yet another, much faster, underlying rhythm of locomotor activity. One generally obscured from running wheel records, so favoured for their ease of collection and quantification, by the large-amplitude overarching influence of the circadian rhythm driving locomotor behaviour and physiology, but a rhythm nonetheless. This ultradian (~ 4 h) rhythm, discussed but rarely investigated, was difficult for us to ignore. In every single experiment where we perturbed the circadian clock in a meaningful way this rhythm was observable. Perhaps it was simply an artefact of removing the circadian system? At least one study, performed using voles, suggested this was not the case; as did the observation of these rhythms in human preterm and neonatal infants. With so little underlying research, and all of it tasked with simply quantifying the behavioural phenotype, it was not until we observed this rhythm in circadian competent mice – using implanted radio telemetry devices while performing an

unrelated study— that we gained enough confidence to begin investigating in earnest; attempting to identify its underlying biological (neurological?) substrate, its purpose and subsequent influence on behaviour and physiology. Therefore, the objective of this second set of investigations was to identify key molecular and neurological underpinnings of this ultradian oscillator in the mammalian brain. We will dedicate the remainder of this dissertation to discussing our specific hypotheses and our preliminary findings surrounding this ultradian oscillator as we believe we have identified its neurological substrate and have significantly narrowed the possible list of molecular mechanism for rhythm generation in mice. Perhaps even more intriguingly, we will finally link our findings in mice to human correlates of the behaviour observed in healthy individuals but also in mental illness, when the oscillator is putatively pushed to great extremes by still unknown factors which are the aetiology of human psychiatric diseases.

Literature review:

The first reported scientific evidence of biological rhythmicity came from plants, specifically the work of Jean-Jacques d'Ortous de Mairan who observed, in 1729, the opening and closing of leaves of the *Mimosa* (1). Importantly, this evidence came not from diurnal studies under the influence of cycles of day and night, but while the plants were maintained in constant darkness so as to remove any possible influence of an external cycle which might drive rhythmicity. Ultimately, this was not a perfect experiment as there were still various other factors like temperature and humidity which might impart external influence but it was a definitive moment in the history of biological rhythms; the first real attempt to prove that this daily dance of the leaves was indeed an endogenous rhythm, one operating on a circadian (from Latin -circa 'about' + -diem 'day') timescale. Indeed, well into the twentieth century, circadian research was dominated by a hunt for correlates of the earth's rotation which might imbue plants and animals with the ability to sense our daily revolutions about the polar axis. Ultimately through careful and deliberate exclusion of these putative factors, coupled with several other pieces of evidence e.g. the observation of free-running rhythms in plants and humans with slightly longer or shorter than 24-hr rhythm generators, the existence of truly endogenous rhythms is a matter of fact (for a fascinating historical review of chronobiology see 2).

In the earlier part of the 20th century, a number of scientists independently began studying rhythmic phenomena in biological organisms e.g. Bünning in botany (3), Garner and Allen in agriculture (4), Richter in mammalian physiology (5), Hastings in protozoology (6), Pittendrigh in biology (7), and both Aschoff (8) and Halberg (9) in medicine. However, it wasn't until the genetic

revolution – marked by the discovery of the *Drosophila* gene *Period* by Kanopka and Benzer in 1971 (10) and followed in the 1980s by the work of Ralph and Menaker identifying a novel mutation in hamsters (*Tau*) with a shorter than usual circadian clock (11) that the molecular underpinning of circadian rhythmicity were explored in earnest. Subsequent advances in molecular biology during the 1990s allowed for the discovery of homologous genes in flies and mice (12-22), identifying the underlying mechanism of circadian rhythmicity: molecular oscillations of transcriptional-translational feedback loops (TTFLs).

Transcriptional-translational feedback times the circadian clock

Molecular clocks can be found in various cells types and tissues throughout the body where rhythmic functioning helps to coordinate specific tasks, like secretion or neurotransmission, while conserving resources and energy. Each molecular clock is driven by a complex system of auto-regulating transcriptional and translational feedback loops with positive and negative elements (Figure 1) (For review see 23). Briefly, the transcription factors *Brain and Muscle Arnt-like protein 1* (BMAL1) and *Clock / Neuronal PAS domain-containing protein 2* (CLK/NPAS2) drive the expression of *Cryptochrome* (CRY) and *Period* (PER) proteins(18, 21, 24-38). These, in turn, suppress their own translation by entering the nucleus and interfering with CLK:BMAL1 dimers bound to Enhancer boxes (Ebox) acting as DNA response elements(39, 40). The time required to complete a single cycle of this negative feedback loop is approximately 24 hours mostly owing to modulation/regulation by several known factors including: *Casein kinase 1 epsilon* (CK1ε) which flags PER proteins for degradation(41-44), *Retinoic acid-related orphan receptors* (RORs) which activate ROR response elements (RORE) found within the CLK and BMAL1 genes(45, 46), and finally the *nuclear receptor subfamily 1, group D* proteins (NR1D, aka REV-ERB or RE) which suppress RORE activation (47-51). Many other key regulators of the transcription, translation and post-translational modifications of canonical clock genes have been identified, each with their own period modulating effects (52-68).

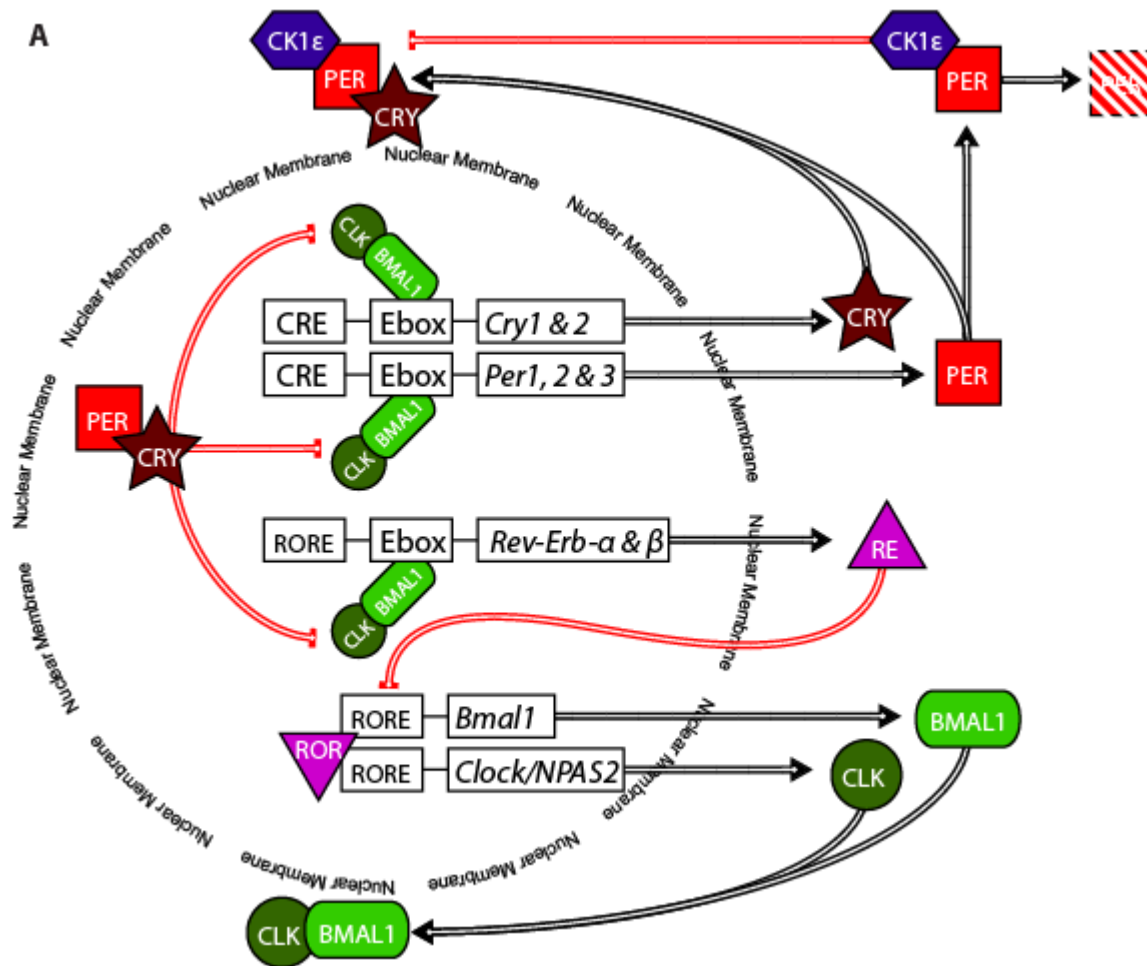


Figure 1: Canonical transcriptional translational feedback loop (TTFL) of the mammalian circadian clock. Black arrows indicate a positive interaction while red arrows indicate negative feedback. For full list of abbreviations see Pg. 10. Figure adapted from (23).

While rhythmic, the oscillations created by these clocks do not necessarily fit perfectly to a circadian (24h) timeframe (69). They require external cues, or zeitgebers (from German -zeit ‘time’ + -geber ‘giver’), to reset and maintain a precisely coordinated system. Without zeitgebers, these oscillators enter a free-run state that is relatively stable within an organism but variable between individuals in a population. The length of a single cycle during free run (denoted as τ) in an individual is related to the biochemical interactions that maintain the oscillations of transcription and translation events in the absence of a resetting mechanism. Even slight allelic variations can affect the rate of activity and/or affinity of any one of these clock proteins, ultimately resulting in minute variations in the frequency of oscillatory mechanisms. Similarly, these same genetic variations lead to individual clocks that differ in their susceptibility to resetting stimuli. The specific mechanism of

action that each zeitgeber may use to interact directly with the molecular clock varies in its site of action. For example, it can reduce the transcription of one clock gene or increase the transcription of another. Other zeitgebers are directly responsible for the physical degradation of clock proteins. In each case, these resetting stimuli interact with the clockwork to transiently shorten or lengthen τ , resulting in phase advances (or delays) in the overall oscillation and eventually synchronizing with the rhythmic presentation of the zeitgeber itself. This process not only targets the master circadian clock but also peripheral oscillators like those found in the stomach and liver. In each tissue type, these clocks are affected by specific zeitgebers that are relevant to the production of clock controlled proteins necessary for the rhythmic functions in these tissues (70).

Not only do the specific zeitgebers that entrain clock processes differ amongst tissues, but there is also differential expression of clock controlled genes. Many proteins in these molecular clocks are coupled to the transcription and translation of clock genes necessary for maintaining oscillations as well as the expression of secondary proteins responsible for the proper functioning of the tissue as a whole (for a recent review see 71). For example, PER1 is a transcription factor for the gene of the renal epithelial sodium channel in murine kidneys (72), and entrainment of these clocks directly influences renal activity in a circadian manner. In the liver, both the time of feeding and intrinsic molecular clocks have been shown to directly regulate the transcription of hundreds of hepatic genes (73). Clock controlled genes (ccgs) encode proteins whose expression oscillates as a result of the distinct transcriptional activity of molecular clock components. This represents a direct site of interaction between ticking of the molecular clocks found in each individual cell and the circadian rhythms of tissue, organ, and central nervous system function. Functions under circadian control include enzymatic processes such as anabolic and catabolic processes (74), gating of hormonal output (75), and even the spontaneous electrical activity of neurons themselves (76). In the liver alone, well over 10% of the expressed gene set are regulated by the molecular oscillator (77) and ultimately 43% of all known protein-coding genes oscillate in the circadian range in at least one organ (78). Furthermore, the existence of variants of these positive and negative elements (PER1, 2 and 3 and Cry 1 and 2 for example) allows for variation in the molecular clock sub-types that are present throughout the body.

Two important features of this molecular clock have been elucidated using a combination of mathematical modeling and experimental approaches, and these features contribute directly to our understanding of self-sustained oscillations of any biological system. The first seemingly ubiquitous

feature, negative feedback, was predicted as early as 1965 to be a plausible mechanism by which biological systems might achieve limit-cycle operation, i.e. rhythmic oscillations (79). This has been observed to be critical to the proper behaviour of the circadian molecular clock using both mathematical modeling (80-82) and experimental approaches (40, 69, 83). But more importantly, negative feedback is a conserved feature of all known biological oscillators found throughout diverse phyla including cyanobacteria, fungi, algae, insects, and plants, and birds (for review see 84). This feature has even been proposed for a newly described clock (85), observed in both a eukaryotic organism and in human red blood cells (86, 87), based on oscillatory redox states which do not rely on transcription of genes at all. While negative feedback is critical for biochemical oscillations of any kind to occur, in a closed system these oscillations will eventually either expand or compress leading to a collapse of rhythmicity. There is a second feature which is necessary for the stability and hence self-sustainability of such oscillatory systems, and more specifically, the timing or intrinsic periodicity of each oscillation: a delay (88). Early mathematical modeling of biochemical processes strongly suggested that the stability of a limit-cycle oscillator, and hence its self-sustainability, was chiefly reliant on a delay component and when several parameters were examined across a wide range of values, the system always collapsed at some basal threshold for the delay regardless of which parameters were adjusted (89). This is also true for electrical systems, whereby the addition of a simple delay line into an analogue circuit rescues stable limit-cycle oscillations from chaotic perturbations (90) or greatly increases the range of stable periods achievable, as well as the sensitivity to periodic input (91). In the mammalian circadian clock this feature is thought to be primarily governed by a built-in delay of the transcription and/or activity of the negative elements. For instance, the *Period* proteins (PERs) are a key node in the regulation of a physiological delay as both their nuclear translocation (41, 61), and cytosolic degradation (92) are strictly controlled and experimental perturbations of the underlying mechanisms governing these processes greatly increases or reduces the intrinsic period of the clock. Similarly, perturbing either the expression or the activity of Cry proteins, also negative elements of the TTFL, are known to alter feedback delays and hence clock periodicity (20, 40). A direct consequence of this strictly controlled delay in the feedback is that it imposes limits on the capacity of oscillatory system to follow zeitgebers which fall outside the range of stable self-sustained periods and hence defines the range of entrainment, bounded by the upper and lower period limits. Any zeitgeber presented regularly but with a timing which falls outside of this range may affect the period, but will not result in stable entrainment to the timing cue. If it is just outside the range, relative coordination may occur, resulting in a scallop

shaped response whereby the rhythm may appear to phase-lock briefly but then break away within one or two cycles (For a concrete example see 93). These points are not trivial, especially if one considers a hierarchical two oscillator system as will be discussed in the latter portion of this thesis. If the entrainment range of the slave oscillator falls, or is pushed, outside of the period shifting capacity of the master oscillator then entrainment may fail and the two may become desynchronized, with chaotic consequences.

As will be discussed later, both negative feedback and a time-delay are critical for producing self-sustained oscillations of any biological system and identifying the underlying molecular or physiological mechanism which subserve these features can be considered crucial for understanding the tic-toc of any biological clock.

Network mediated functions of the suprachiasmatic nucleus: a collection of 20,000 molecular clocks

The primary circadian pacemaker of the central nervous system of mammals is found in the SCN of the anterior hypothalamus. It was first identified by hypothalamic electrolytic lesioning experiments (94-96). Efferent projections and humoral signals produced by the SCN entrain circadian oscillators in other parts of the brain and body (97-99). Furthermore, the SCN modulates neuronal processes essential for rhythmic behaviours and physiology such as arousal, body temperature, and locomotion (100).

Historically the SCN, a bilateral collection of nuclei with approximately 10,000 neurons per side, are subdivided into two sub-nuclei: a dorsomedial shell and a ventrolateral core, based on retinal innervation patterns and distinctive cell phenotypes (101). More recently, it has become increasingly apparent that these subdivisions are not strictly appropriate. Genetic tools allowing us to further dissect heterogeneities in neuropeptide expression and anatomical connectivity demonstrate that the distinction between core and shell are much more blurred than previously hypothesized (102). Importantly there are a number of neuropeptides expressed within subpopulations of the SCN (for review see 103) and we, and others, propose that these might truly represent (as opposed to the gross anatomical subdivisions) the functional units of the SCN. These units might be responsible for carrying out a number of distinct roles within the pacemaker network including sensitivity to incoming zeitgeber inputs, rhythm generation and maintenance in the face of perturbation, and rhythmic and coordinated output of circadian signalling. We will briefly discuss

these distinct functions – inputs, central generators, and outputs – in terms of the responsible neuroanatomical and humoral pathways.

To date, three major afferent pathways have been elucidated for the SCN: the glutamatergic and pituitary adenylate cyclase-activating peptide (PACAP) releasing retinohypothalamic tract (RHT) (104, 105), the neuropeptide Y (NPY) secreting geniculohypothalamic tract (GHT) (106), and serotonergic (5-HT) projections from the dorsal raphe nucleus (DR) (107). Each distinctly contributes to the net photic input to the SCN, ultimately impinging upon intracellular processes through second messenger signalling. Specifically, cellular activation engages downstream effectors to phosphorylate cyclic-AMP responsive elements binding proteins (CREB) which bind to CREB response elements (CREs) found in the promoters of the mammalian *Period* genes (27, 108-110). In this way light input can directly modulate the oscillations of TTFs found within each SCN neuron ultimately shifting the internal timer to a new light dark cycle or responding to single pulses of light.

A large question in circadian biology is the precise manner in which the individual molecular oscillators found within each cell can create coordinated rhythms in locomotion, hormone secretions, body temperature, and many other physiological processes. Primary axonal outputs of the SCN include GABAergic (γ -aminobutyric acid) efferents to the paraventricular hypothalamus (PVN), dorsomedial hypothalamus, and sub-paraventricular zone (sPVZ) (111). Each of these hypothalamic nuclei in turn integrates this rhythmic signal with homeostatic and humoral signals conveying them to structures responsible for circadian modulation of physiology (112, 113). As an inhibitory neuromodulator these SCN efferents ultimately suppress neuronal activity within the hypothalamus and beyond in anti-phase to the electrical activity of the SCN itself (114), at least in nocturnal rodents. Secondary SCN efferents include axonal projections from PVN endocrine neurons like those responsible for corticotropin releasing hormone which directs the HPA axis, while others target descending autonomic projections to preganglionic parasympathetic neurons of the brainstem and sympathetic neurons of the spinal cord and these include both glutamatergic and gabaergic signalling (99, 115-118) which, for example, stimulate peripheral melatonin secretion from the pineal gland. However, the SCN does not solely communicate via axonal release of classical neurotransmitters. A number of diffusible signals also carry rhythmic information. Critically, at least for locomotor rhythms, this was demonstrated by the implantation of an encapsulated fetal SCN which conferred behavioural rhythmicity to its host even in the absence of axonal outputs (119, 120). Among these signals – whose expression also delineates the putative functional units discussed

above – are the humoral neuropeptides arginine vasopressin (AVP), vasoactive intestinal peptide (VIP), and gastrin releasing peptide (GRP). Yet others are released in smaller quantities such as tumor necrosis factor alpha (TNF- α), cardiotrophin-like cytokine (CLC), neuromedin S (NMS), and prokineticin 2 (PRK2)(121-125). These two types of signals, axonal and humoral, should not necessarily be defined as completely separate in function as there are at least two examples where neurotransmitters and endocrine signals converge to ultimately time SCN output to target tissues (126). While each of these neuropeptides has been implicated in some way in the outputs of individual molecular oscillators of the circadian clocks, their role as functional units within the master circadian pacemaker is only now coming to light.

The last 30 years has seen significant advancements to our understanding of the SCN as the hierarchical pacemaker within a body-wide network of tissue and function specific circadian clocks. Most of these advancements concern themselves with critical physiological inputs and behavioural/physiological outputs, treating the SCN itself as a “black box” structure capable of integration and output, with little regard for the biological underpinnings of such capabilities. There is mounting evidence indicating that network architecture (i.e. functional connectivity) within this brain region is solely responsible for both the precision (with respect to 24 hour periodicity) and the robustness (with respect to amplitude and phase resetting) of the master circadian pacemaker. It is currently held that the intercellular coupling of these neurons provides the substrate (network) from which all pacemaker properties are derived. The SCN itself is composed of thousands of individual oscillators which behave similarly to every molecular oscillator within the body when dispersed in culture (127); exhibiting a wide range of intrinsic periods (20-28 hours) and a large inter-period variability with no significant phase-coupling between individual oscillators (128). Furthermore each individual oscillator exhibits discontinuities of phase response curves (PRC) to zeitgeber input (type zero resetting). When studied in vivo by observing running wheel behaviour and simultaneous multi-unit electrical activity (129), or using organotypically cultured SCNs in vitro to preserve the network connectivity (130), individual oscillators pheno-copy the properties of the master pacemaker itself. This strongly suggests that the network connectivity of the SCN is responsible for several canonical pacemaker properties including a narrow range of periodicities, phase coupling between oscillators, increased amplitude of molecular oscillations (and electrical activity) and a significantly altered range of entrainment which directly impacts on the phase response curves generated by pulsed input presented around the clock. Most interestingly, a recent study using organotypically cultured SCNs

demonstrated that even in the absence of a functional molecular clock, due to the disruption of *Bmal1*, a gene encoding an essential protein of the oscillator, the SCN can exhibit stochastic oscillations (albeit very infrequently) within the circadian range which are not observed in either dispersed cells or in cultured SCNs when intercellular communication is disrupted pharmacologically (131). This same property is attributed to the network in *Cry* knockout SCNs whereby circadian rhythmicity is retained in the face of a cell-autonomous circadian defect (132). These studies demonstrate that rhythmicity can arise as an emergent property of the SCN network itself, even in the absence of cell-autonomous circadian oscillators, and suggests a level of circadian organization and pacemaker capacity inherent in the network itself.

Importantly the exact character and contribution of individual nodes in this network are unknown. There are many neuropeptides expressed within SCN cells and these subpopulations occur with varying degrees of overlap and varying proportions of unidirectional and reciprocal innervation within the SCN itself (133). Some subpopulations of note express AVP, VIP, and GRP to name just a few (see Figure 2). We propose that neuropeptide signalling might distinctly contribute to this network architecture, identifying key nodes in the network, and allowing us to use genetic tools to specifically label and interact with these nodes in a meaningful way to probe their necessity and sufficiency for different SCN-mediated functions with a particular emphasis on the control of locomotor activity in mammals.

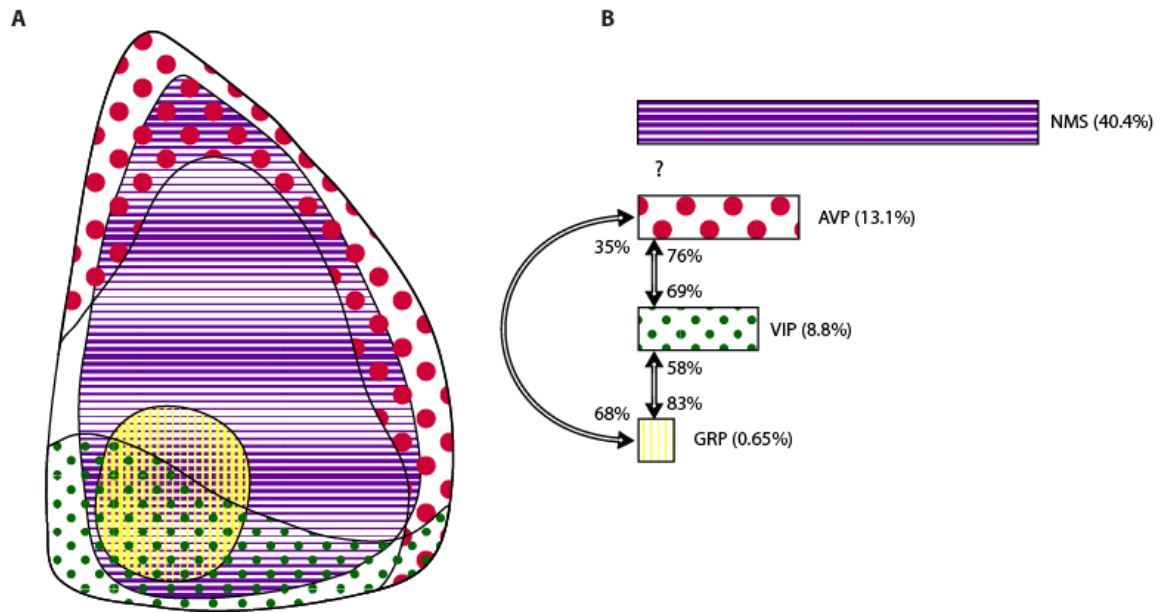


Figure 2: Approximate distribution of neuropeptide expressing cells within the rodent suprachiasmatic nucleus. **(A)** Distribution of cell bodies based on immunofluorescent localization of neuropeptide expressing cells including Neuromedin-S (NMS, purple lines), Arginine Vasopressin (AVP, red dots), Vasoactive Intestinal Peptide (VIP, green dots), and Gastrin Releasing Peptide (yellow lines). **(B)** Distribution estimates of reciprocal connectivity based upon immunofluorescent apposition in rat (black arrows and adjacent percentages) as well as total population estimates from mice (percentages in brackets). Note that reciprocal connectivity between NMS and other populations is currently unknown (?). Adapted from (133), (134) and (135).

Clocks underlying rest-activity cycles

Historically, the primary method used to investigate circadian rhythmicity in mammals has been locomotor activity behaviour as recorded by counting revolutions of a running wheel provided within the cage (136). To this day, most investigations of the circadian clock be they pharmacological, behavioural, or genetic use running wheels as the standard method of recording locomotor activity, however several other methods have been described including: floor circuits completed by skin conductance (137), infrared beam breaks (138), overhead infrared sensors (139), and implantable telemetry devices (140). While these other methods may in some way be considered equivalent as they record movement in general – including stereotyped behaviour such as grooming or slight movements related to brief periods of arousal even during sleep – running wheel activity represents a significantly higher threshold of arousal and may also represent an additional rewarding and reinforcing behavioural component. Indeed, locomotor activity can feedback onto the circadian clock itself and alter behavioural rhythmicity (for review see 141). This is not to say that this method should not be considered, and indeed it provides robust and reliable recordings of circadian

rhythmicity. This cautionary note is rather intended as a possible explanation for why only the circadian component of the rest/activity cycle has been studied in depth, while other periodicities underlying locomotor activity rhythms have been relatively ignored.

There are currently four distinct rhythms in the rest/activity cycle in mammals which have been observed, studied, and at least partially characterized in terms of their neural substrate. The first has already received an extensive introduction, and will be referenced throughout this thesis as THE classical example of rhythmic rest/activity control: the 24-hour rhythm controlled by the circadian system. Not surprisingly these rhythms are the most prominently observable and when quantified, consistently shows the highest spectral density across a range of hours to days. There are currently two other rhythms of the rest/activity cycle which have been observed in the circadian range: food anticipatory activity (FAA) the behavioural manifestation of a food-entrainable oscillator (FEO)(142) and methamphetamine-sensitive circadian oscillations (MASCO) (143). While the neural correlates of the underlying oscillators are currently unknown it is very well established that these rhythms are controlled by neural processes which are independent of the known circadian clockwork both in terms of genetic substrate (144, 145) and anatomical location (146) despite the their periods typically falling within the circadian range. Most interestingly, these rhythms are only detectable under very specific physiological conditions with FAA being robustly observed only after several days of entrainment to a single daily meal - although there is some evidence that rats may entrain to multiple meals per day (147, 148)- and the MASCO being only observed after several days to weeks of ad libitum access to methamphetamines, a potent drug of abuse known to cause sustained arousal (146). Of note, the earliest work using methamphetamines and studying locomotor rhythms suggest that while at the highest doses the MASCO oscillates with a circadian, or even infradian (>24hr) rhythm, the effects on period appear to be dose dependent (see figure 4 in 149). This observation, as we will hopefully demonstrate, is a cornerstone to understanding of the origins of the MASCO which appears to represent a particular manifestation of yet another oscillator, one potentially operating at shorter than circadian timescales.

Ultradian rhythmicity of locomotion across species

The rhythmic output of this final oscillator, whose identification and description is the major focus of my thesis, falls within the ultradian range with a period of approximately 4 to 6 hours. This rhythmicity has remained generally ignored by circadian researchers, and this is likely because it is

not easily discernable in running wheel records made in intact animals. It appears to be more readily detectable by other means of locomotor activity assessment (e.g. monitoring ambulatory activity employing telemetric implants) Using this method we, and others, have frequently observed three equidistant peaks of locomotor activity in intact mice during the night – when mice are most active— and this modulation appears to ride on top of the circadian driver of locomotor activity. Upon disruption of the circadian system however, whether by lesioning the suprachiasmatic nucleus or genetically ablating the circadian clock it becomes immediately apparent (12, 21, 150). In order to assure the reader that this is not simply a byproduct of breaking the circadian clock, these rhythms are indeed quantifiable in intact mice, although some rather sophisticated signal analysis processing may be required in order for them to be extracted. Indeed analysis of this kind was performed in nine different strains of mice and all showed ultradian rhythmicity in this range (151). Furthermore, this ultradian periodicity is not restricted to mammals but was first described in *Drosophila melanogaster*, the common fruit fly, when key genetic components of the circadian clock were first being perturbed to assess function (152). Interestingly, rhythmic activity within this frequency range is also prominently observed in pre-term human infants, prior to the development of a fully functioning circadian system (153-156). It has also been observed at the level of stereotyped behaviour in cases of mental retardation, as well as in the social synchronization of a small tribe of indigenous people native to Colombia (for review see 157). Of the four rest-activity rhythms observed in mammals, the ultradian rhythm is the least well characterized, and indeed only the handful of studies mentioned above currently exist which attempts to describe these rhythms behaviourally, with no evidence presented for either molecular or (neuro)anatomical substrates.

A central aim of the proposed research described herein was to elucidate a putative oscillator underlying these ultradian locomotor behavioural rhythms, both mechanistically and anatomically. Here we will provide the first evidence of such an oscillator in the brain (158) and go on to further anatomically and molecularly define this second major oscillatory system which, in conjunction with the central circadian pacemaker, drives our daily rest-activity patterns. Importantly, the proposed research may also advance our understanding of the two inducible behavioural oscillators, the FEO and the MASCO. In fact, our findings suggest that the MASCO represents a long-period manifestation of the ultradian oscillator we discovered while the involvement of dopamine in the FEO is under direct and critical scrutiny by several groups at this very moment (159-161).

Specific Aims:

Aim 1: We posit that the selective genetic ablation and resurrection of the circadian molecular clock in specific subpopulations of cells within the master pacemaker can help to elucidate their contributions to the network properties of the suprachiasmatic nucleus including: responses to zeitgeber inputs, rhythm generation and maintenance, and behavioural and physiological outputs. We generated mice selectively lacking or specifically expressing circadian clocks (critically relying on *Bmal1* expression) within vasoactive intestinal peptide expressing cells of the SCN and then tested these animals for both behavioural and physiological alterations. Furthermore, we investigated mice expressing firefly luciferase driven by the circadian transcription of *Bmal1* in order to visualize network interactions. We hypothesized that the loss of circadian clocks with VIP+ cells, and whose release is critical for SCN synchrony, should similarly result in a loss of circadian rhythmicity under constant darkness but still allow for the entrainment of locomotor rhythms by light.

Aim2: Mice lacking circadian control of locomotor rhythms do not exhibit static levels of arousal and locomotion. Rather they show alternations between quiescence and locomotor activity which are strikingly similar to the patterns observed in both invertebrates lacking circadian control and in preterm and postnatal human infants prior to the development circadian rhythmicity. We hypothesized that these alternations were actually oscillations driven by a novel ultradian (<24h) oscillator in the mammalian brain. We first characterized these behavioural oscillations, demonstrating endogenous and self-sustained rhythmicity. We then tested this oscillator system's response to arousal-modulating perturbations, both genetic and pharmacological, correlating the endogenous rhythms with specific molecular oscillations in vivo. Finally, we confirmed our results and localized this novel oscillator to specific neurocircuitry using a virally-mediated and genetically-selective chemogenetic approach to drive neuronal activation within this circuit.

Aim3: Having previously mapped out at least one node of the circuitry necessary for ultradian rhythmicity, we decided to follow the roadmap of clockwork dissection laid out for us by the study of other biological clocks, chiefly the circadian system. We hypothesized that by manipulating specific molecules and circuits within the brain we might perturb critical feedback loops, observing period changes critically implicating these targets as necessary for rhythm generation and/or maintenance. By employing the previously developed behavioural model for most accurately quantifying ultradian locomotor rhythmicity – ablating the circadian clock and removing exogenous timing cues – we set about to test several candidates by either genetically or pharmacologically blocking putative feedback signalling.

Chapter 2: VIP-expressing cells of the SCN act as an autonomous pacemaker but do not drive behavioural rhythmicity

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Abstract:

The last 30 years have seen significant advancements to our understanding of the suprachiasmatic nucleus (SCN) as the hierarchical pacemaker within a body-wide network of tissue- and function-specific circadian clocks. Most of these advancements concern themselves with critical physiological inputs and behavioural/physiological outputs, treating the suprachiasmatic nucleus itself as a “black box” structure capable of integration and output, with little regard for the biological underpinnings of such capabilities. There is mounting evidence indicating that network architecture (i.e. functional connectivity) within this brain region is solely responsible for both the precision (with respect to 24 hour periodicity) and the robustness (with respect to amplitude and phase resetting) of the master circadian pacemaker. Furthermore, diversity of neuropeptidergic expression within suprachiasmatic nucleus subpopulations suggests a functional heterogeneity of cells within the pacemaker network. Vasoactive intestinal peptide (VIP) is expressed in a subset of suprachiasmatic nucleus cells and its production, and release, have been previously associated with entrainment to light and maintenance of pacemaker synchrony. Here we demonstrate -ex vivo- that this subpopulation of cells is also capable of intrinsic rhythm generation and self-synchrony, identifying it as a distinct and independent pacemaking population. Interestingly, mice lacking functional clocks outside of this subpopulation fail to demonstrate behavioural rhythmicity or light entrainment. This strongly suggests that the output of circadian clocks is mounted within another population of cells, either within the suprachiasmatic nucleus or external to it, is responsible for behavioural rhythmicity. Therefore circadian clocks within VIP-expressing cells are capable of endogenous circadian rhythm generation but they are not sufficient for behavioural expression of this rhythmicity.

Introduction:

All mammals exhibit circadian (daily) patterns of behaviour and physiology regulated by a complex network of endogenous clocks and coordinated by the master pacemaker in the suprachiasmatic nucleus of the hypothalamus (SCN) in order to phase align them to the solar day (for review see 162). Since light input cannot reach every tissue in the body directly, there is a necessity for a hierarchical organization of information processing from the master SCN pacemaker to central and peripheral cellular clock networks (reviewed in 163). The SCN represents a collection of small, densely packed neurons which have been historically subdivided into two sub-nuclei: a dorsomedial shell and a ventrolateral core. This is based on retinal innervation patterns and phenotypically distinctive cell types (101). Each sub-nuclei contains approximately 10,000 neurons distributed bilaterally just above the optic chiasm and to either side of the third ventricle, with individual cells displaying oscillations of cellular, biochemical, and genetic processes. These oscillations are driven by a complex system of auto-regulating transcriptional and translational feedback loops (TTFL) with positive and negative elements (23) and while rhythmic, do not necessarily fit perfectly to a circadian (24-hr) timeframe (69) nor do they all oscillate within the exact same temporal window. They require extrinsic timing cues, called zeitgebers (from German – zeit ‘time’ + geber ‘giver’), to reset and maintain a perfectly coordinated system. Without zeitgebers, oscillators may enter a free-run state, with reduced synchrony but also increased peak to peak variability within individual cells as well as increased period length variability across populations of cells.

While the core and shell represent distinct anatomical regions within the SCN, the use of this terminology severely oversimplifies the complexity of the neural network (164). Indeed there are a number of neuropeptides expressed by distinct (or sometime overlapping) subpopulations of SCN cells that are heterogeneously distributed and spatially diffuse (103). Numerous functions have been attributed to one or another of these subpopulations including photic and non-photic input, intrinsic rhythm generation, and even behavioural or physiological output (reviewed in 165).

In support of functional segregation based on neuropeptidergic content, it has recently been proposed that two distinctly localized subpopulations, vasoactive intestinal peptide positive cells (VIP+) in the core and arginine vasopressin positive cells (AVP+) in the shell, play an important role in the inter-cellular coupling of these neurons with neuropeptide release acting as local zeitgebers to synchronize the population of oscillators contained within the SCN (82). Interestingly,

these two population are reciprocally enervated (133). It is therefore proposed that the reciprocal effect of these two populations is capable of coordinating the precision, robustness, and stability of rhythmic output of SCN in order to control physiological and behavioural outcomes. In support of this idea, the translation and expression of these neuropeptides are themselves directly influenced by the transcriptional loops of the molecular clock in the case of AVP (26) or by rhythmic electrical activation in the case of VIP (166). It is currently held that the intercellular coupling of these neurons, and others within the SCN, provides the substrate (network) from which all pacemaker properties are derived. The SCN itself is composed of thousands of individual oscillators which behave similarly to all other TTFLs within the body when sufficiently dispersed in culture; they exhibit a wide range of intrinsic periods (20-28 hours) and a large inter-period variability (128). However, when studied *in vivo*, by observing running wheel behaviour and simultaneous multi-unit electrical activity (129) or using organotypically cultured SCNs *in vitro*, thus preserving the network properties and connectivity between clock neurons (167), the oscillators of the individual SCN neurons pheno-copy the properties of the master pacemaker as a whole. This strongly suggests that the systems-level network connectivity of the SCN is responsible for several canonical pacemaker properties including a narrower ranges of periodicities, phase coupling between oscillators (i.e. synchrony), increased amplitude of molecular/electrical oscillations, and a significantly altered range of entrainment and phase response curve, all of which confer robustness (168).

Previous experimental evidence suggests that the AVP-expressing dorsomedial shell is a major rhythm generating population (169), and evidence from receptor knockout mice showing perturbed locomotor rhythmicity (170) and susceptibility to phase shifting stimuli both *in vivo* and *in vitro* (171). Therefore, we wanted to test the sufficiency of this population of cells for rhythm generation more explicitly by selectively resurrecting the circadian clocks solely within these neurons.

Conversely, little is known about the contribution of VIP+ cells to pacemaking properties. Initially it was proposed that the VIP+ population in the ventrolateral core existed as gatekeepers of photic input only, as the individual clocks within these cells are generally considered weakly rhythmic but respond strongly to light stimulation, shifting much more rapidly than the dorsomedial shell (172) or indeed any peripheral oscillators (173). In support of this role, *Vip* transcription is upregulated in response to calcium influx and/or GPCR activation (cAMP response elements – CRE), and neurotrophin signalling (cytokine response element – cyRE) via cis-acting regulatory regions in its promoter sequence (174), making its synthesis distinctly sensitive to neuronal input.

There are at least four putative E-boxes within the promoter region. Some have been identified as sites for constitutively active myocyte enhancer factor-2 (Mef2) binding necessary for appropriate cell specificity (175). However, none of them represent a canonical E-box reserved for circadian clock mediated transcription (176) as expression of *Vip* mRNA is unchanged in Clock mutant mice (177). However, there is still the distinct possibility that intracellular circadian regulation may affect VIP release in some way (178). Evidence that both the core and the shell of the SCN can tick independently and in antiphase under specific conditions (179), and that rhythms of AVP and VIP can be dissociated in vitro (180) suggests that there is indeed a pacemaking subpopulation in the ventrolateral, SCN, one which might rely upon VIP+ cells (but not necessarily VIP itself) as these are the major constituents in this SCN region, and this might be revealed by manipulating the circadian clocks within the VIP+ cells of the ventrolateral core.

More recently, another role of this population has been elucidated, that of maintaining synchrony within and among the circadian clocks expressed throughout the SCN. By manipulating VIP signalling, several groups found that the circadian control of locomotion was severely compromised, but importantly not abolished. This was true for both the genetic disruption of *Vip* (181-183) as well as its receptor (181, 184, 185). Furthermore, at the system level, ex vivo observations of bioluminescence within the SCN network also demonstrates a severe disruption of synchrony whereby individual cellular clocks, while still rhythmic, are no longer appropriately phase locked or synchronized (169, 182).

Finally, there is a question of output. How is it that the SCN and the thousands of clocks found within, can communicate with the rest of the mammalian brain to control the plethora of behavioural and physiological processes under circadian control? For example, VIP peptide expression is important for circadian regulation of glucocorticoids (186). Another example of such process, and one that is of great import to most circadian biologists as it remains the most easily collected and quantified, is locomotor activity. Furthermore, both transplant (187, 188) and inducible *Clock*-mutant expression (189) clearly demonstrate it is the SCN-genotype that determines locomotor period. What role might the distinct neuropeptidergic subpopulations within the SCN play in conveying and/or modulating the signals regulating the drive to locomote? Again, the work of Dr. de la Iglesia in rats suggests that both the core and the shell are capable of driving locomotor activity, but whether each rhythm is intrinsically generated/maintained, is untestable in the desynchronized model owing to the necessity of an imposed 22-hr L:D cycle. To address this open

question we decided to study and manipulate the VIP and AVP cells of the SCN, as they represent the dominant constituents of the core and shell regions, respectively

How might the role of specific neuropeptide populations within the SCN be tested? Several groups have gone after the peptides themselves, as discussed above, generating animal models with loss of either peptide or obligate receptors. While this may be informative for our understanding of those peptides, there is still the distinct possibility that these subpopulations communicate using classical neurotransmitters released at synapses (190, 191), or even gap junctions (192, 193) and manipulations of neuropeptide expression may fail to catch perturbations in the network that are distinctly marked by neuropeptide content but not reliant on peptidergic communication. Furthermore, there are many other brain and peripheral tissues which express these peptides, ones which might have very little to do with rhythm generation, synchrony, or rhythmic output *per se*, but could still influence our physiological and behavioural readouts when globally and developmentally perturbed. Therefore, we chose instead to manipulate the circadian clocks within these neuropeptidergic populations using Cre-*loxP* technology (194, 195) with the assumption that any perturbations in circadian locomotor rhythmicity would be attributable specifically to cells within the master circadian pacemaker and not off target effects. This strategy has been recently adopted for this exact purpose, to study the role of AVP (196) and Neuromedin-S (135) neuronal subpopulations within the SCN with several manipulations performed including conditional ablation and conditional mutation of circadian clocks within these cells, and in the case of neuromedin-S, blocking synaptic transmission.

Using recently validated AVP and VIP driven Cre-recombinase lines of mice in conjunction with two floxed mouse lines. One with *loxP* sites flanking Exon 8, a critical domain of Brain and Muscle Arnt-like 1 (BMAL1) and one with *loxP* sites flanking a stop cassette inserted upstream of the BMAL1 gene, allowing for a two-pronged approach, one in which BMAL1 was functional in all but a select subset of cells where Cre-recombinase was present, and one in which the entire animal was devoid of cellular clocks except in the Cre-recombinase expressing cells. We therefore tested for both the necessity and the sufficiency of the circadian clock machinery within these neuropeptidergic populations with regard to intrinsic rhythm generation, intracellular synchrony, and locomotor output control.

Results:

Cre-recombinase mediated excision of Bmal1

In order to assess the contribution of circadian clocks within AVP and VIP expressing cells of the SCN we generated two lines of mice by genetically crossing BAC transgenic AVP-Cre^{Tg} (197) and VIP-IRES-Cre mice (*Vip*^{Cre/np}) (191, 198) with mice harbouring *loxP* sites flanking a critical exon (#8) of the *Bmal1* gene (*Bmal1*^{flx/flx}, Figure 3A) (199) to generate mice selectively lacking circadian clocks within this subpopulations of cells (ACKO and VCKO, Figure 3B). While both Cre lines have been previously validated, we crossed in a reporter line with *loxP* sites flanking a stop codon upstream of a fluorescence reporter protein sequence, TdTOMATO (*TdTomato*^{fls/fls}) (200), in order to verify selective expression, and activity, of Cre-recombinase within the AVP+ dorsomedial shell and the VIP+ ventrolateral core of the SCN (Figure 3C). Initial attempts to excise *Bmal1* from both AVP+ and VIP+ cells was woefully unacceptable (<30% recombination, data not shown), therefore we furthered our efforts by using mice which were homozygous for VIP-IRES-Cre and carried a flx-BMAL1 allele ('flx' allele) over a conventional *Bmal1* KO allele ('-' allele) genes (a strategy effective for increasing recombination efficiency in 201). Unfortunately, due to the transgenic (and random) insertion of AVP-Cre, we were not able to achieve homozygosity for the *Avp*-Cre transgene and, likely owing to that, were again met with disappointing recombination rates even after producing 'floxed over knockout' (*Bmal1*^{flx/-}) mice. Therefore we chose to abandon this particular line and focused on the manipulation of the VIP+ population only. Furthermore, due to the increased cost and reduced breeding efficiency required, we abandoned the simultaneous presence of the TdTomato reporter allele within our mouse models, but of course this left us unable to directly quantify the recombination efficiency as attempts to label with anti-Cre were not satisfactory and anti-VIP staining does not present with strong and faithful cytoplasmic labeling, even after colchicine injections. However, visual inspection of anti-VIP and anti-BMAL1 labeling demonstrated a distinct lack of BMAL1 in the ventrolateral core of the SCN and few, if any, cell bodies labeled by anti-VIP colocalized with BMAL1 in any of the sections we examined suggesting efficient recombination (Figure 3D).

Loss of Bmal1 in VIP-expressing cells produces unstable entrainment and alters phase response curves

These mice were then placed in running wheels allowing for the assessment of a variety of circadian parameters including light entrainment, free running period under constant conditions, and

phase responses to light pulses given at various phases of the circadian cycle. Under 12:12 L:D conditions conditional knockout mice (VCKO mice) did not demonstrate any impairments in period, general cage activity, phase angle of entrainment, or length of the alpha band ($p>0.05$), however in a measure of entrainment, the least-squares error of a fitted line to the onsets of activity, these animals significantly differed. This can be observed in the relative coordination (scallop) of the activity onsets about the light to dark transition (Figure 4A) and when quantified represents more than a five-fold change in the error (Figure 4B) suggesting that while entrained, the daily precision of the circadian timing system may be impaired. Upon release into constant darkness, we also observed a significant decrease (mean difference $\pm$ SEM = 0.4553 ± 0.1052) in the period of conditional knockout mice as well as a continued difference in the least-squared error of the onsets (Figure 4A and C). Given the role of the SCN ventrolateral core, and more specifically VIP+ cells, in gating photic input, we assessed phase responses to light pulses (30min, 200lux) at different phases of the circadian cycle (Figure 4D). Interestingly, phase advancing light pulses (CT15) were approximately half as effective while phase delaying pulses (CT21) had a greater magnitude of effect for conditional knockouts as compared with their wildtype counterparts (Figure 4E) with the overall effect that light pulses were more effective at phase advances than delays, the opposite of a normal murine light-induced PRC.

Selective resurrection of circadian clocks within VIP+ but not AVP+ cells

By developing a novel mouse line with a *loxP*-flanked stop-cassette inserted upstream of the *Bmal1* transcriptional start site (*Bmal1*^{fls/fls}, Figure 5A) we were able to test not only the necessity of circadian clocks within neuropeptide expressing cells but also their sufficiency for normal locomotor output generation. By crossing this floxed-stop line with VIP-IRES-Cre line we were able to generate mice which lacked circadian clocks bodywide with selective resurrection within VIP+ cells, however resurrection attempts in AVP+ cells using AVP-Cre^{Tg} failed entirely (Figure 5B). Further investigation using in situ hybridization against *Avp* in both wildtype and *Bmal1*^{-/-} and *Bmal1*^{fls/fls} mice revealed no detectable *Avp* mRNA in the SCN of clock dead animals (Figure 5C). This strongly suggests that transcriptional activity at the AVP promoter requires BMAL1. Intriguingly, this was the case for *Avp* within the SCN only, as normal expression levels were observed in other AVP+ regions like the paraventricular hypothalamus and the supraoptic nucleus in clock dead mice. Unfortunately for us, the lack of transcriptional activity also meant that Cre-recombinase was

extremely unlikely to be expressed in the SCNs of these mice, making excision of the flx-stop cassette, and hence resurrection of BMAL1 expression impossible.

Thankfully, our attempts to resurrect the clock in VIP+ cells were a success. As confirmed with immunofluorescent labeling of BMAL1, mice homozygous for the flx-stop *Bmal1* allele showed absolutely no clock gene expression in the SCN (Figure 5D), or indeed in any brain expressing VIP including the cortex, hippocampus, amygdala, and periaqueductal grey (data not shown). Heterozygous VIP-IRES-Cre mice on the other hand had strong BMAL1 expression in the ventrolateral core of the SCN indicating successful resurrection of the TTFLs in those cells (Figure 5D). This expression exclusively comprised the region where VIP expression is normally distributed in the SCN and when the total number of BMAL1 positive cells was compared to TdTomato positive cells from our original validation attempts (Figure 3C) we found there was no significant difference between the levels of reporter and clock gene expressing cells strongly suggesting efficient recombination (Figure 5E).

Ex vivo oscillations of luciferase reveal synchronous, high amplitude circadian oscillations in VIP+ cells

While the absence of BMAL1 strongly predicts the absence of circadian clock gene oscillations at the cellular level, its presence does not sufficiently demonstrate that these cells indeed exhibit normal rhythmicity. Therefore, we crossed in a third strain transgenically expressing the firefly luciferase gene driven by the *Bmal1* promoter sequence (*Bmal1*-Luc^{Tg}, Figure 5B). The presence of this transgenic insertion allows for the visualization of endogenous circadian clock rhythmicity at the single cell level using a custom built bioluminescence imaging platform (Figure 6A and B). With this mouse line, we confirmed that the clocks resurrected within the SCNs of *Vip*^{Cre/wt} *Bmal1*^{fls/fls} *Bmal1*-Luc^{Tg} mice (VSB) did indeed significantly oscillate (Figure 6C and D). Meanwhile, cells within the dorsomedial shell of the SCN, which was illuminated by constitutive activity of the BMAL1 promoter driven luciferase at all times of day, did not show significant oscillations throughout the seven days of recording. Furthermore, cosinor fit waveforms of the significantly rhythmic cells showed large amplitude oscillations similar to those of intact mice (Figure 6E and F), and these oscillations remained synchronized throughout the recording although there does appear to be a distinct subpopulation of cells (~25%) in the VSB mice which is slowly desynchronizing with a longer period (Figure 6G and I). However it must be noted that this second population was only observed in one of the three mice tested. In general, the phase-distribution of rhythmic cells was

preserved over the entire recording in both wildtype and VSB mice (Figure 6H and J) also indicating strong synchrony amongst cellular clocks. Of note, the observed periods were significantly longer in VSB mice (Figure 6K), which is in direct opposition to, and consistent with, the shorter endogenous period observed in mice with selective deletion of clocks with VIP+ cells (Figure 4C).

Intriguingly, while resurrection of circadian TTFLs in VIP+ cells produced a pronounced, high-amplitude and highly synchronous oscillation of clock genes, these animals were arrhythmic on the behavioural level when studied under constant conditions (Figure 7A). Furthermore, analysis using continuous wavelet transforms to assess the dynamics of circadian oscillations over the entire experiment (Figure 7B and C) demonstrate not only a dampened amplitude of the diurnal variation in locomotor activity under L:D conditions, but also a precipitous drop immediately after the L:D→D:D transition (Figure 7C and D) suggesting that these mice have severe perturbations in the circadian drive to locomote. Indeed, there is no statistical difference in locomotor activity rhythm amplitude in the circadian range between mice without clocks bodywide and those with selective resurrection in VIP+ cells, either in L:D or D:D (Figure 7E). Because there was some variability in the levels of BMAL1 resurrection in the SCNs of these mice, we ensured that this was not a contributing factor by assessing the correlation between resurrection and circadian amplitude. Indeed there was no significant correlation between Chi-Squared amplitudes at 24h and the number of BMAL1 positive cells, strongly supporting the complete lack of circadian locomotor rhythmicity observed visually and quantified using the wavelet analysis.

Discussion:

Cre-mediated ablation and resurrection of Bmal1

While we were able to successfully ablate *Bmal1* expression from VCKO mice, we did not achieve acceptable results for ACKO mice. This is paradoxical in some ways as the transgenic insertion of Cre, as opposed to an internal ribosomal entry site knockin (IRES) generally has the potential to yield much higher rates of expression (194). This may be due to higher endogenous levels of transcriptional activity at the *Vip* promoter as opposed to *Avp* promoter although this has never been quantified directly to our knowledge. It is unlikely the result of discrepancies in developmental expression of these two genes as they are both expressed around embryonic day 18 (165). Furthermore, the *Avp*-Cre line was capable of producing sufficient cre-recombinase in AVP+ cells in order to express *Rosa26* driven TdTOMATO (data not shown), clearly suggesting that it may

be an issue of accessibility of the *Bmal1* genomic locus, or more specifically exon 8 of *Bmal1*. We can rule out a universal issue with the *Bmal1*^{flx/flx} line as this has been successfully used for various cell types in the periphery (74, 199), the brain (202), and even the SCN (135, 201), although in the case of *Synaptotagmin10* and our own experience with *Vip* did require two copies of the Cre allele and hemizygous flx-*Bmal1* for efficient SCN recombination. Reduced accessibility to the *Bmal1* loxP sites within the SCN could be due increased presence of transcriptional machinery acting upon this gene, interfering with excision. This idea supported by the high amplitude expression of the mRNA and the protein product of *Bmal1* specifically within the SCN. Of note, recent study successfully used AVP-Cre^{Tg} mice to excise *Bmal1* however it is clear that the iCre line (improved Cre-recombinase) was generated in house (196). This highlights the need, at all times, to properly validate recombination efficiency whenever using a newly generated Cre line, even if the floxed line has previously been successfully used in other contexts.

This naturally leads to a discussion of our inability to resurrect *Bmal1* in AS mice. One possibility is that the same issues are at play, with a lack of accessibility to the *Bmal1* promoter. However there are several arguments against such a case. First, the site of our loxP-flanked stop-cassette is in a different genomic location within the *Bmal1* locus. The second, the design is very similar to the flox-stopped design employed for *TdTomato*, which showed efficient recombination in the *Avp*-Cre mice (data not shown) and in that case it is knocked in to the Rosa26 promoter, one of the most transcriptionally active constitutive promoters in the whole mammalian genome, strongly arguing that it is not the accessibility which is at fault. Finally, a reduction in recombination efficiency, as seen in the ACKO mice, still resulted in at least some excision whereas we did not identify even a single neuron expressing BMAL1 in the SCNs. The most parsimonious explanation therefore is a complete lack of transcriptional activity at the *Avp* promoter in SCN cells congenitally lacking BMAL1. The reasoning is as follows: if activation of the *Avp* promoter relies – either directly or indirectly—on BMAL1 transcriptional activity and these mice never express BMAL1, then they will not produce *Avp* nor will they express Cre-recombinase driven by the exact same promoter sequence, regardless of where it lies in the genome. While we can only offer circumstantial evidence at this time, in the form of a complete lack of *Avp* expression as assessed by in situ hybridization (this lack occurs specifically within the SCN and not the nearby PVN or SON, Figure 5C), we are confident in our conclusions based on reduced (but not abolished) SCN specific *Avp* expression in *Clock*^{-/-} mice reported by several groups (26, 177, 203). Interestingly, the SCNs of *Per1/Per2* double-

mutant mice still have quantifiable rhythms of AVP release *ex vivo* (204), strongly suggesting that it is BMAL1 which is strictly required for *Avp* transcription.

Given the issues raised above, it might be tempting to dismiss the lack of behavioural rhythmicity observed in VS mice due to a lack of efficient rescue. Ultimately we were hoping to address this question definitely by comparing and contrasting any phenotypic differences between AVP+ and VIP+ resurrection. In lieu of this, we quantified and compared the number of BMAL1 resurrected cells to the total possible pool of Cre expressing cells and found no significant difference (Figure 5D). Furthermore there was absolutely no correlation between the number of resurrected cells and the rhythmic amplitudes observed regardless even when discounting the significance of the amplitude (Figure 7G). Finally, since we do not observe any qualitatively discernable change in VIP protein expression or the number of VIP expressing cells in these mice (Figure 3D), we feel confident to suggest that BMAL1 resurrection in VIP+ cells are a significant enough population that should these cells truly control locomotion, they would be able to do so in our resurrected mice.

The role of VIP-expressing cells in the suprachiasmatic nucleus

As VIP+ cells receive direct photic input (134, 178, 205), it could be argued that the onset error might simply be due to altered masking responses to light, but that would only account for earlier onsets occurring during the light period. Visual inspection shows that the onsets also frequently begin well after the light to dark transition, further strengthening the argument that it is the daily precision of the circadian timer, and hence daily phase resetting (i.e. entrainment) which is perturbed in these mice. Of note, the continued difference (but lower variance) observed in this parameter after release into constant darkness suggests that both entrainment AND endogenous pacemaker precision are independently impaired in these mice. Is this latter effect possibly due to a lack of intracellular synchrony? This is a distinct possibility as similar observations were made in both *Vip*^{-/-} (183) and *Vipr2*^{-/-} mice (184) which have a distinct lack of synchrony between cellular oscillators *ex vivo* (169). While the phase of endogenous oscillations under LD do not significantly differ in these mice (data not shown), there is a significant difference in the immediate leftward shift (~ 30min) of locomotor onsets after release into constant darkness (Figure 4E) suggesting that either photic entrainment or more specifically photic gating might be impaired in these mice. This suggests, albeit much less strikingly than the almost 12h phase advances observed in VIP neuropeptide (183) or receptor (181) knockout mice, that the TTFLs themselves within this

population of cells does play a role in photic gating of incoming light input. This is further confirmed by the novel observation, possible only because of our strategy of deleting the TTFLs, that the phase response curve is significantly and specifically perturbed in these mice. If the VS mice, on the other hand, had any observable circadian rhythmicity it could have been interesting to test the sufficiency of clocks within VIP+ expressing cells for photic gating, but as such we could not perform this experiment.

The idea of VIP+ cells as a pacemaker population is an interesting one, and counter to most discussion surrounding these oscillators to date, especially given previous research suggesting that the ventral portions of the SCN are weakly rhythmic compared to those of the whole or dorsomedial SCN only (206). The high amplitude and highly synchronous rhythms observed here are themselves surprising given reports that clock gene expression in these cells is highly variable and only one third of this population is rhythmic in sufficiently dispersed cultures (207). Importantly however, it is unlikely that VIP+ cells represent the sole driving force for rhythm generation as these SCN cultures also show AVP and other neuropeptide expressing cell types can generate intrinsic rhythms of clock gene expression when similarly dispersed. It does strongly suggest however that network mediated coupling amongst VIP+ TTFLs plays a key role in the observed synchrony and amplitudes in our VS slices.

The observation of significantly shortened Tau in our VCKO mice is consistent with our own observations ex vivo, with resurrected clocks exhibiting a period phenotype of approximately one hour longer than wildtypes. One would expect that the removal of a long period population from a network within a widely distributed range of observed periods (82), would indeed result in the lowering of the period of the whole oscillator network as predicted. Interestingly, both mathematical modeling and in vitro experiments of oscillator interactions suggests that in a mixed population it is the slower oscillators that have a higher impact on the ultimate speed of the network (208) bear this in mind as it will become important in a moment. While the period of VIP+ resurrected clocks is longer, it is not as exaggerated as those seen in dispersed SCN cells of *Clock*^{Δ19} heterozygous mice (33), although the neuropeptidergic lineage of the reported cells is unknown but presumably mixed. The reason we bring up the allele in particular is that a recent paper showed a long period phenotype in mice with conditional expression of this gene in neuromedin-S neurons but when they also performed the same experiment in VIP+ neurons they reported no period difference. It may have been interesting for them to then test *Vip-Cre Clk*^{Δ19/Δ19} as homozygosity

should result in an equivalent phenotype to that demonstrated here with the VCKO mice. Ultimately though, we must still attempt to explain why a loss of clocks in VIP+ cells resulted in a phenotypic period change in our experiment when none was observed in the VIP-Cre *Clk*^{Δ19} mice. One would almost expect the opposite to be the case. Perhaps a clue resides in the character of the clocks within this particular subpopulation, it is still unclear why or how the periods of individual oscillators is so varied although there are some theories as to why that may be advantageous (130, 209, 210), but it remains that this range of periods exists and not just in single cells but within the network of the SCN itself (206). Regardless, our results strongly argue for a conserved, robust, and highly synchronized long-period TTFLs within the ventrolateral core, and more specifically the VIP+ cells as demonstrated here; pushing the question of the regulation of period encoding in SCN subpopulations to the fore and placing the answer squarely at the systems-level.

The most parsimonious conclusion is that a direct output of circadian TTFL, residing within another population of cells, either within the suprachiasmatic nucleus or possibly external to it, is responsible for driving circadian locomotor rhythms. There are of course multiple arguments for the population residing within the SCN as transplant (187, 188), inducible Clock-mutation (135, 189), and quasi SCN-specific clock ablation (135, 201, 211) all determine the period of locomotor rhythmicity. The most likely candidate for a diffusible factor driving locomotor rhythms is *Prk2* (123, 212) as direct i.c.v. infusion of this protein has marked effects on circadian control of running wheel activity. The expression of *Prk2* is directed by both light and the circadian clock (212) and *Prk2* receptor KO mice have severely blunted locomotor rhythmicity (213) with no apparent deficits in the master circadian pacemaker *ex vivo*. Interestingly, while severely perturbed, there does appear to be at least some circadian expression of locomotor activity in these mice, suggesting *Prk2* is not the sole output of circadian locomotor control.

Conclusions:

The relatively mild behavioural phenotype observed in the conditional knockout mice is in stark contrast to those observed in either the peptide or receptor knockout models and may suggest that our recombination efficiency was not 100%. Regardless the phenotype is relatively unsurprising given that VIP expression does not rely on the circadian clock *per se*, as it does for AVP, but rather is driven by activation of these cells by GPCR and electrical signalling. In support of this we continue to see similar VIP labelling in SCNs of VCKO mice. What then, if any, is the specific role of the circadian clock within VIP+ cells? Why not dispense with them completely? From our VIP+ TTFL

resurrection experiments, clearly the circadian clocks in these cells are functional, precise, and highly synchronized, strongly suggesting a conservation of T⁺TFL function and hence some evolutionary advantage. At least one answer to this question has been proposed previously: gating of photic input to prevent aberrant phase shifting.

Potentially countering this argument is the recent work of Fan et al. which demonstrated that VIP+ neurons do not show a day-night difference in either spontaneous or optogenetically stimulated firing frequency (191). Something to consider however, is that there does appear to be a day night difference in firing frequency after excitation at laser powers below 50 microwatts (although this was not statistically tested). Furthermore there is no strict necessity for electrical activity to oscillate *per se*, as the control of quantal release is sufficient to produce alterations in signalling strength in the absence of a change in vesicular fusion frequency. This entire point might be moot if VIP release occurs somatodendritically or in an action potential independent manner. These considerations, taken with the fact that the spontaneous firing frequency is being measured *in vitro* with absolutely no extra SCN input (as opposed to *in vivo*), suggests that these cells might indeed oscillate both in spontaneous firing frequency (under conditions of stochastic network input) and in their responses to endogenous stimulation of a more appropriate strength; by retinal glutamate or pituitary adenylate cyclase-activating polypeptide, for instance. This idea is supported by the work of Brancaccio et al. demonstrating that both intracellular calcium and cAMP response element binding protein (CREB) transcription fluctuates in synchrony with circadian clock gene expression within the SCN and that GPCR activation of VIP+ cells using DREADDs has the ability to shift the subsequent T⁺TFL spatiotemporal dynamics in a manner similar to that of stimulating the entire circuit (198). Our own investigation using a floxed-*Bmal1* model allows us to conclusively state that T⁺TFLs within VIP+ cells are indeed necessary for the appropriate response to photic input however their question of their sufficiency remains unresolved.

Furthermore the work of Brancaccio et al. suggests that these cells are also necessary for plastically responding to circuit input, shaping network properties like period and amplitude over several days/cycles, dissipating transiently generated inertia slowly over time. This is supported by our own observations of phase response curve anomalies in mice lacking the clocks in these cells. Perhaps the best analogy to use for the role of VIP+ cells in this context is a mechanical clutch: storing some applied force (zeitgeber input) in a flywheel (T⁺TFLs of VIP+ cells) and then gradually releasing that energy by applying torque to a mechanical load (phase shifting of the other T⁺TFLs in

the SCN) until both systems are in sync (synchrony of all TTFLs resulting in circadian entrainment). Why the circadian system would require a clutch-like mechanism for entraining to large shifts in the timing of solar day is a question that has been long asked, especially in the context of jet-lag and shift-work. To date, the most satisfying answer presented is simply that it buffers the entire system against fluctuations of light-levels inherent in our environment – from indoor-outdoor (or burrow-foraging) transitions to cloud-cover with hourly/daily shifting weather patterns. A system too directly coupled to light levels would not perform well in the face of strong day to day, or even dawn to dusk, photon flux. This argument has been made for the circadian system as-a-whole numerous times and given the data presented here, we feel it is most appropriate to pin the distinct function of zeitgeber entrainment squarely on the TTFLs within the VIP+ cells of the SCN, at least in so far as they respond to specific photic and non-photoc inputs (214).

We feel that these experiments satisfy two distinct sets of goals towards elucidating the relative contributions of individual neuronal subpopulations to the system-level network properties of the SCN (215). The first is to confirm the role of VIP+ cells in this context and further characterize the role of TTFLs within these cells. The second, and we feel the more important, is to develop the tools and lay out the methodological roadmap for investigating the function of the other distinct neuropeptide populations within the SCN. This includes the elimination of TTFLs, as others have done for both AVP+ and NMS+ neurons, but also the selective resurrection of these clocks and the investigation of their sufficiency for both behaviour and network interactions *ex vivo*.

Materials and Methods:

Animals *Bmal1*^{-/-} (21), *Avp-Cre*^{Tg} (QZ20Gsat, 197), *Vip-IRES-Cre* (tm1(cre)Zjh, 216), *TdTomato*^{fls/fls} (200), *Bmal1*^{fls/fls} (199), *Bmal1*^{fls/fls}, and *Bmal1*-luc (217) mice are all congenic strains backcrossed on a C57BL/6J genetic background. Animals were housed under 12hr:12hr Light:Dark cycles (LD) unless otherwise stated. Experimental procedures were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the McGill University Animal Care Committee.

Aschoff's type II phase response curves In order to probe the effects of light stimulation on the master circadian pacemaker we performed 4 time-point phase response curves. These were collected according to Dr. Aschoff's originally proposed protocol (218) and the rationale for performing them as such is elucidated by Dr. Mrosovsky and others (219-221). Briefly, mice were entrained for two weeks to LD upon which point they were released into constant darkness (DD) and subjected to

either no light pulse (free-run) or a 30 minute light pulse of 200 lux (measured at cage bottom) at one of four timepoints (CT 15, 21, 3, or 9) on the first day in DD. The animals were then allowed to free run for one week in order to measure any change of phase caused by the light pulse. Phase shifts were calculated using the discrepancy between the measured activity onsets before and after the light pulse (Clocklab, Actimetrics). These shifts were then corrected by subtracting the original shift measured for each animal after release into DD (with no light pulse) in order to account for any potential effects of light masking on the activity onsets measurements under LD.

Locomotor activity Animals were individually housed in light-controlled cabinets and activity was recorded continuously (ClockLab, Actimetrics). Actograms, displaying binned running wheel revolutions per 6 minutes (0.1 hr), and the associated Chi-squared periodograms, displaying amplitude, were generated using ClockLab software.

Bioluminescent Imaging Collection of single cell resolution bioluminescent imaging was performed using a custom designed bioluminescent imaging platform. Briefly, a black plexiglass box fitted with a baffled ventilation system was constructed inside of a passive incubator (BD400, Binder). Inserted through the top of this box is a 60mm tube terminated by a 20x long working distance objective (NPLM20X, Union). Attached above is a supercooled CCD camera (Ikon M, Andor) with liquid coolant (25% Ethylene Glycol, 75% dH₂O) pumped into the incubator/camera from an liquid chiller (Oasis 160, Solid State Cooling Systems) using PVS tubing. Within the box a fully automated stage is constructed using two linear 100mm actuators (M403.42S, Physik Instrumente (PI)) and a vertical translation stage (NT55-032, Edmund Optics) fitted with a 10mm linear actuator (M228.10S, PI). The stage is operated with three DC stepper motor controllers (C663, PI) attached via USB to a windows 7 computer running MikroMove software (PI). Imaging was controlled and captured using Andor Solis (v4.19.30001.0, Andor) and then saved as .tiff stacks for import into MATLAB for custom processing and analysis.

Circadian amplitude determination of running wheel activity In order to most accurately estimate the circadian amplitude of VSB mice in constant conditions we calculated the chi-squared periodogram (Clocklab) for the first seven days in D:D conditions. The highest amplitude value observed between 22 to 24-hrs was taken as the estimate.

Continuous wavelet transforms To visualize and quantify the dynamics of circadian period and amplitude, continuous wavelet transforms using the Morlet wavelet (222) were performed for the 20

to 28-h range using custom algorithms written in Matlab (v2013b, Mathworks). Locomotor activity data of 14 consecutive days across the L:D to D:D transition. For each spectrogram, a ridge identifying the peak amplitude of the spectrum was generated as described (223) and the associated values were used to demonstrate a significant drop in circadian amplitude upon removal of light cues from the environment.

Imaging and analysis Image stacks were first pre-processed in image J software (National Institute of Health) in order to rotate and crop the bilateral SCN (259 px²). Each stack was then individually run through custom written Matlab software (v2013b, Mathworks) in order to automatically extract and analyze circadian bioluminescent signals across the entire SCN slice. First, the image stack was processed using a 3 timepoint median recursive filter for each pixel to eliminate any sensor noise or cosmic rays which would only be in a single image. This was followed by a 2D convolution in order to better center the signal of each individual cell and reduce the number of cells which might overlap. The SCN lobes were then traced by hand in order to define our region of interest and avoid the inclusion of bioluminescent rhythms from any neurons falling either in the surrounding hypothalamus or 3rd ventricle. A 6x6 pixel grid was overlayed on top of the images based on the average cell diameter measured in image J and the median intensity of the pixels within each quadrant over time was taken as the signal. Each signal was then detrended using a 2nd order polynomial and followed by a 2.5h lowpass butterworth filter. In order to identify significantly rhythmic quadrants a best fitting cosinor in the 20 to 28-hr range was calculated for each signal with a zero-amplitude F-test performed at an alpha level of 0.05. Significantly rhythmic signals were then remapped back onto each image for visual identification of rhythmic cells and these signals were used for plotting the data to visualize synchrony, phase, and period of the entire recording.

Immunohistochemistry All Immunohistochemistry was performed using cryoprotected (30% Sucrose), free-floating coronal sections from fixed brains collected after intra-cardiac perfusion (4% paraformaldehyde). Cryostat (Leica) sliced sections were divided into 4 serial sets of 40µm slices and were immediately stored in a cryo-protectant solution at negative 20°C until processed for immunocytochemistry (224). Immunofluorescence: was carried out as previously described (225). Briefly, after blocking endogenous antibodies, sections were incubated overnight at room temperature with an antibody directed against VIP peptide (1:500 rabbit anti-VIP, Cedarlane). After rinsing, they were secondary incubation was performed for 2h (1:250 goat anti-rabbit Alexa488, Invitrogen). Immunofluorescence was imaged by darkfield microscopy (AxioObserver Z1, Zeiss) in

order to visualize VIP protein and genetically expressed TdTomato. Co-localization was performed using the cell counter plugin of ImageJ (National Institute of Health). *DAB labeling*: was carried out as previously described (226). In short, brain sections were rinsed with 5x5min in cold phosphate buffer (0.1M PB; pH 7.3), incubated for fifteen minutes in a quenching solution (1% H₂O₂ in 0.1M PB), rinsed three times in cold 0.1M PB, followed by a blocking solution (0.3% Triton-X detergent and bovine serum albumen at 0.01g/mL in 0.1M PB) for thirty minutes at room temperature, and finally incubated for 48h at 4°C with a primary antibody raised against the c-terminal BMAL1 peptide (1:10,000 Rabbit Anti-BMAL1, Novus). After incubation with the primary antibody, sections were rinsed (5x5min. in 0.1M PB) and incubated for one hour with an anti-rabbit secondary antibody (Biotinylated goat anti-rabbit IgG H+L 1: 250; Vector Labs) diluted in 0.1M PB containing Triton-X and BSA. The sections were incubated (3x5min. in 0.1M PB) at RT for one hour with the avidin-biotinylated enzyme complex (ABC Complex; Vector Labs). Sections were again rinsed (3x5min. in 0.1M PB) and incubated in a solution of 3,3'-diaminobenzidine (DAB; 5mg per 17mL of buffer; Sigma) and Nickel Chloride (1% w/v), and developed for 4 min. using 25µL of a 0.1% H₂O₂ solution. After final rinsing, sections were wet-mounted onto statically charged slides (SuperFrost Plus), dehydrated in three consecutive series of alcohols, defatted (Clearene) and coverslipped with Permount (Fisher). DAB Immunocytochemistry was imaged by brightfield microscopy (AxioObserver Z1, Zeiss). Quantification of expression was performed on a single medial section of the SCN using the cell counter plugin of ImageJ (National Institute of Health) in order to estimate the amount of BMAL1 resurrection (N=6 per genotype).

In-situ hybridization In situ hybridization was performed as previously described (121). Briefly, following decapitation, brains were removed and quickly frozen at -80°C. Serial coronal hypothalamic brain slices (25µm) were collected using a cryostat and stored at -80°C until hybridization. Sections were hybridized overnight at 60°C to a digoxigenin-labelled riboprobe targeting the coding regions of mouse *Arp* (nucleotides 16-459 of the *Arp* mRNA, Genbank NM_009732.2).

Organotypic cultures Extraction and organotypic culturing of SCN tissue was carried out using solutions and techniques as described by Dr. S. Yamazaki in a review paper written for *Methods in Enzymology* (227). Briefly, the animal was euthanized by cervical dislocation and the whole brain was quickly extracted and submerged in ice cold Hanks's Buffered Saline Solution before being sliced on a vibratome (V1200s, Leica) at a thickness of 250µm. A whole slice containing the SCN was then

microdissected under a stereomicroscope (Leica) in order to extract the bilateral SCN (approx. 1mm²) which was then transferred onto a membrane insert (PICMORG50, Millicel) in a 35mm petri dish with 1ml of recording medium and a final concentration of 0.1mM of luciferin (VivoGlo™ D-Luciferin Salts, Promega). Finally, the tissue was hermetically sealed within this dish using a 40mm round coverslips (Size 0, Harvard Apparatus) using vacuum grease (14-635-5D, Dow-Corning) and the dish was transferred into the custom built bioluminescence platform for recording of cellular resolution bioluminescence over 1 week.

Statistical analysis GLM and repeated measures ANOVA, t-tests, and linear regression analyses were performed using Prism 5 (GraphPad). All circular statistical analysis was performed using custom written Matlab software (v2013b, Mathworks).

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Figures:

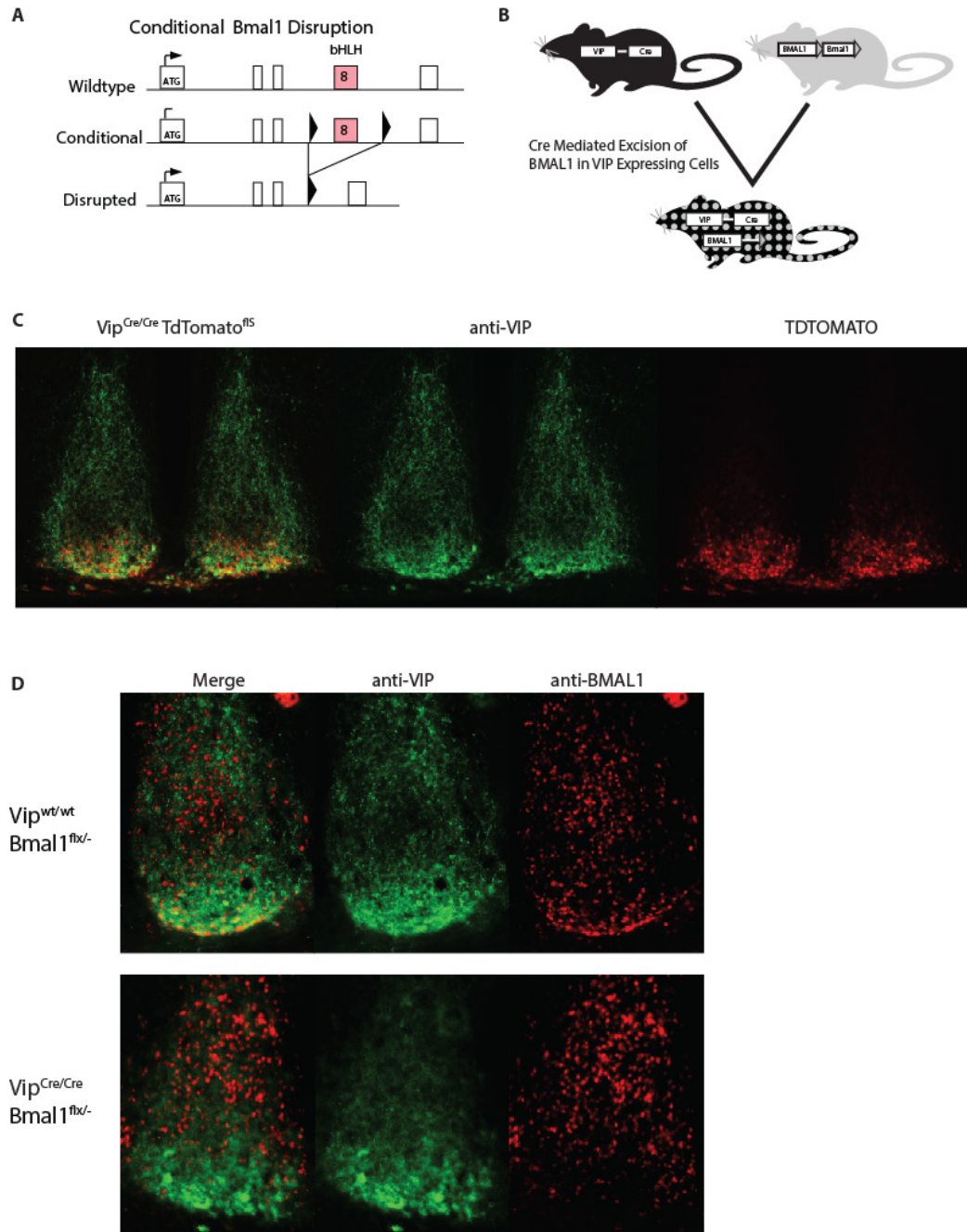


Figure 3: Cre-recombinase mediated excision of *Bmal1*. **(A)** Conditional disruption of *Bmal1* transcription is achieved via loxP sites flanking Exon 8, resulting in a frameshift truncation and subsequent loss of function. **(B)** Breeding scheme of conditional knockout of BMAL1 in VIP-IRES-Cre mice. **(C)** Verification of Cre-mediated excision in *Vip^{Cre}* TdTomato^{fls} (VT) mice using a genetically encoded fluorescent protein, TdTOMATO (red) shows distinct colocalization with immunolabelling of VIP (green) in the ventrolateral core of the SCN. **(D)** Cre-mediated excision results in a loss of BMAL1 protein expression (red) specifically within VIP immunolabelled cells (green).

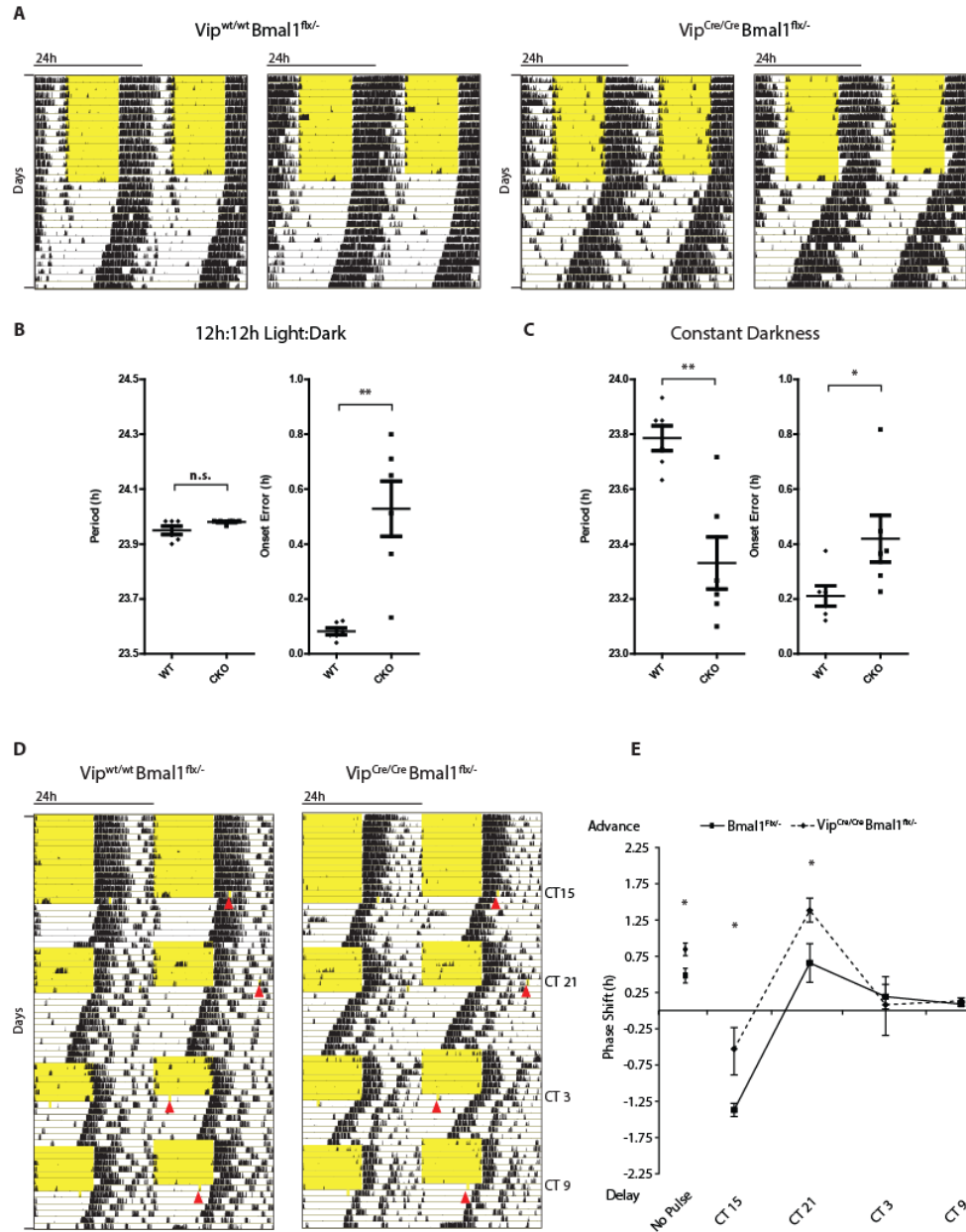


Figure 4: Behavioural consequences of the loss of circadian clocks in VIP+ cells. **(A)** Representative running wheel actograms of *Vip^{Cre/wt} Bmal1^{flx/-}* (VCKO) mice and their wildtype littermates under 12:12 LD (yellow bars) and constant darkness. **(B)** Quantification of period and onset error reveals a greater than five-fold increase in onset error under a light dark cycle in VCKO mice (mean $\pm$ SEM; N=6; $t_{10}=4.391$, $^{**}P<0.005$, unpaired t-test). **(C)** Constant darkness revealed a significant difference in both free-running period (mean $\pm$ SEM; N=6; $t_{10}=4.330$, $^{**}P<0.005$, unpaired t-test) and onset error (mean $\pm$ SEM; N=6; $t_{10}=2.240$, $^{*}P<0.05$, unpaired t-test). **(D)** Representative running wheel actograms of VCKO and wildtype mice during light pulse experiments (30 min at 200lux, red arrows). **(E)** Quantified phase response curves calculated from light pulse experiments demonstrate a significant dampening of phase delays (mean $\pm$ SEM; N=6; $t_{10}=2.249$, $^{*}P<0.05$, unpaired t-test) and significantly increased phase advances (mean $\pm$ SEM; N=6; $t_{10}=2.326$, $^{*}P<0.05$, unpaired t-test) in VCKO mice, however the typically observed “dead zone” remained unperturbed. All PRC values presented and analyzed are individually corrected for the significant genotypic difference in shifting after release into constant DD (mean $\pm$ SEM; N=6; $t_{10}=2.724$, $^{*}P<0.05$, unpaired t-test) as proposed by (219).

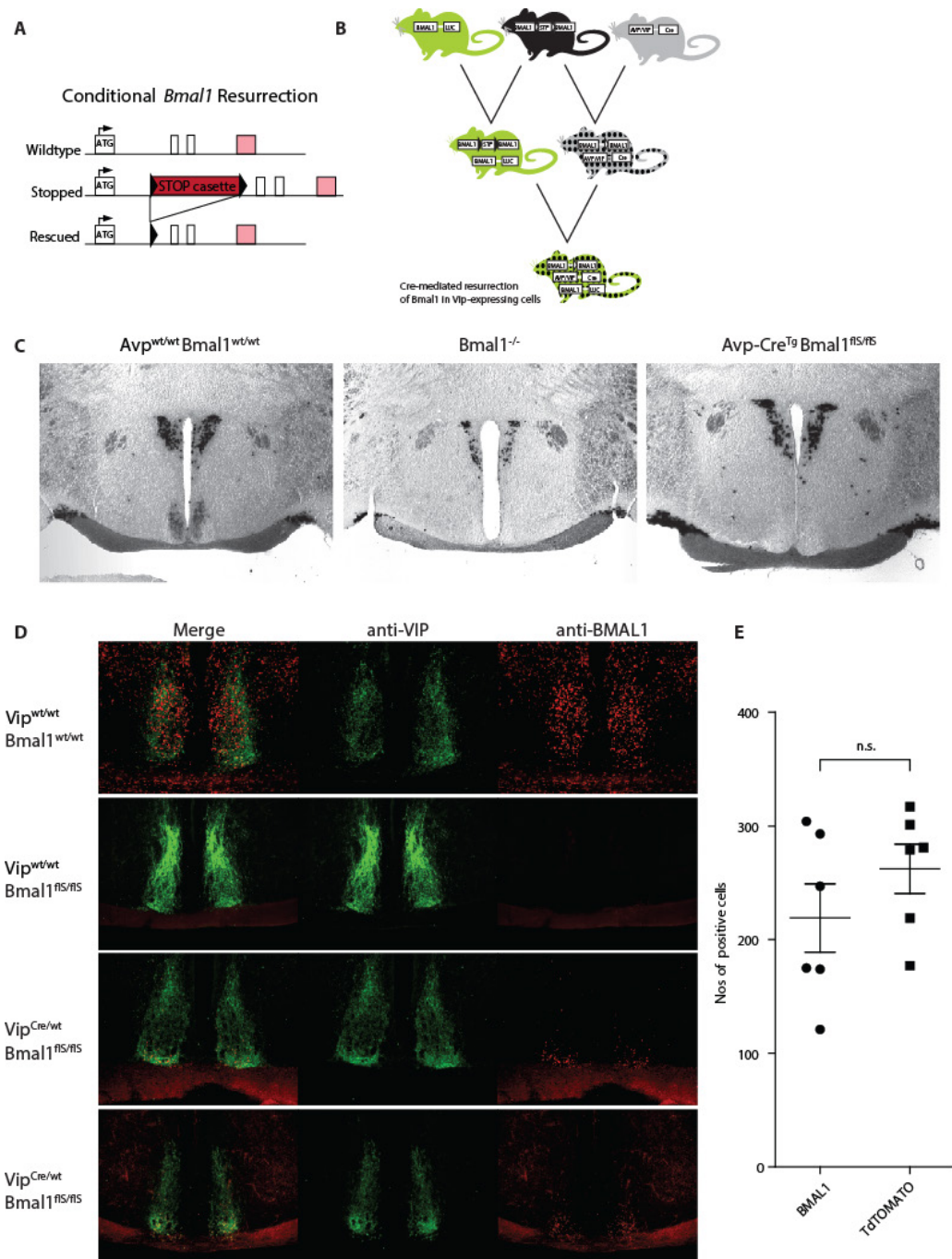


Figure 5: Cre-recombinase mediated resurrection of BMAL1. (A) Conditional resurrection of *Bmal1* transcription is achieved via excision of loxP sites flanking a stop codon upstream of the *Bmal1* gene (*Bmal1*^{fls/fls}). (B) Breeding scheme of conditional rescue of BMAL1 in AVP-Cre^{Tg} (AS) and VIP-IRES-Cre mice (VS) and the inclusion of *Bmal1*-luciferase reporter for bioluminescent imaging. (C) DIG-labelled *in situ* hybridization against *Avp* in *Bmal1*^{-/-} and AS mice suggests that failed resurrection is the direct result of SCN abrogated *Avp* transcription due to a congenital loss of BMAL1 expression. (D) Conditional resurrection in the SCN of VS mice as demonstrated by immunolabelling colocalization of VIP (green) and BMAL1 (red). (E) Quantitative comparison of the number of BMAL1 expressing cells in VS mice against the number of TdtTOMATO expressing cells in VT mice reveals no significant difference in recombination efficiency (mean ± SEM; N=6; t₁₀=1.168, P=0.27, unpaired t-test).

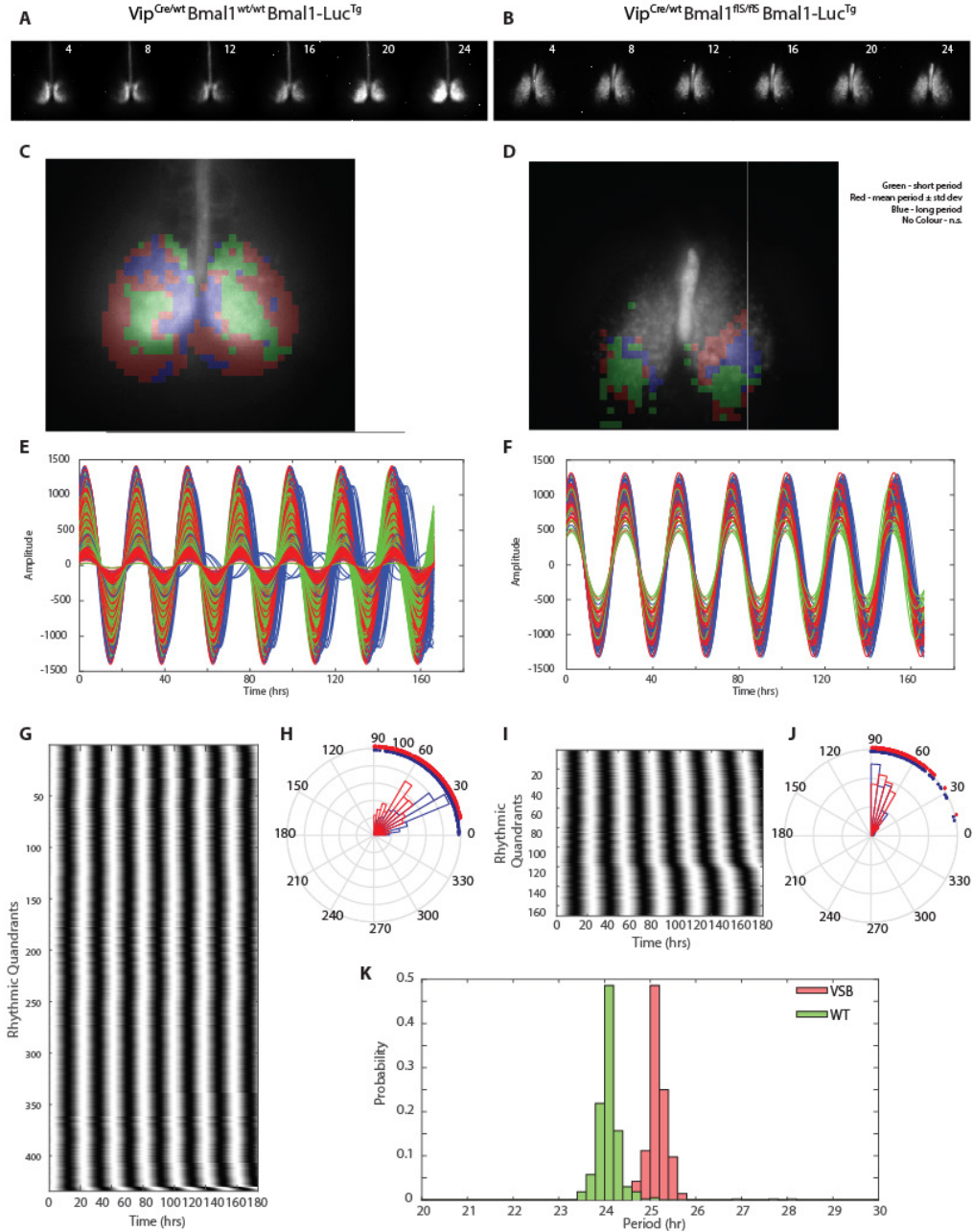


Figure 6: Bioluminescence imaging reveals self-sustained, high amplitude, highly synchronous oscillations in VIP+ subpopulation of the SCN. (A, B) Recording of single cell resolution bioluminescent imaging over a single circadian cycle reveals typical travelling wave of peak luciferase activity from SCN shell to core in wildtype littermates (A) but only oscillations within the ventrolateral core in VS mice (B). (C, D) Cosinor analysis of grid overlay confirms significant rhythmicity (coloured quadrants, $P < 0.05$, bonferroni corrected F-test) solely in the core region of VS (D) mice (colours representing period distribution according to the legend). (E, F) Modeled cosine waveforms for all significant quadrants illustrate the high amplitude and synchronous oscillations observed in the SCN core of VS mice (F). (G to J) Normalized plots of waveforms sorted by period demonstrate phase synchrony in WT and VS mice (G, I) and polar phase distribution plots (polar projected histogram and circumferential dots) of the first (blue) and last (red) oscillation demonstrate that synchrony does not differ over time in either genotype (H, I). (K) Analysis of period distribution reveals lengthening of τ in the molecular clocks of VS mice.

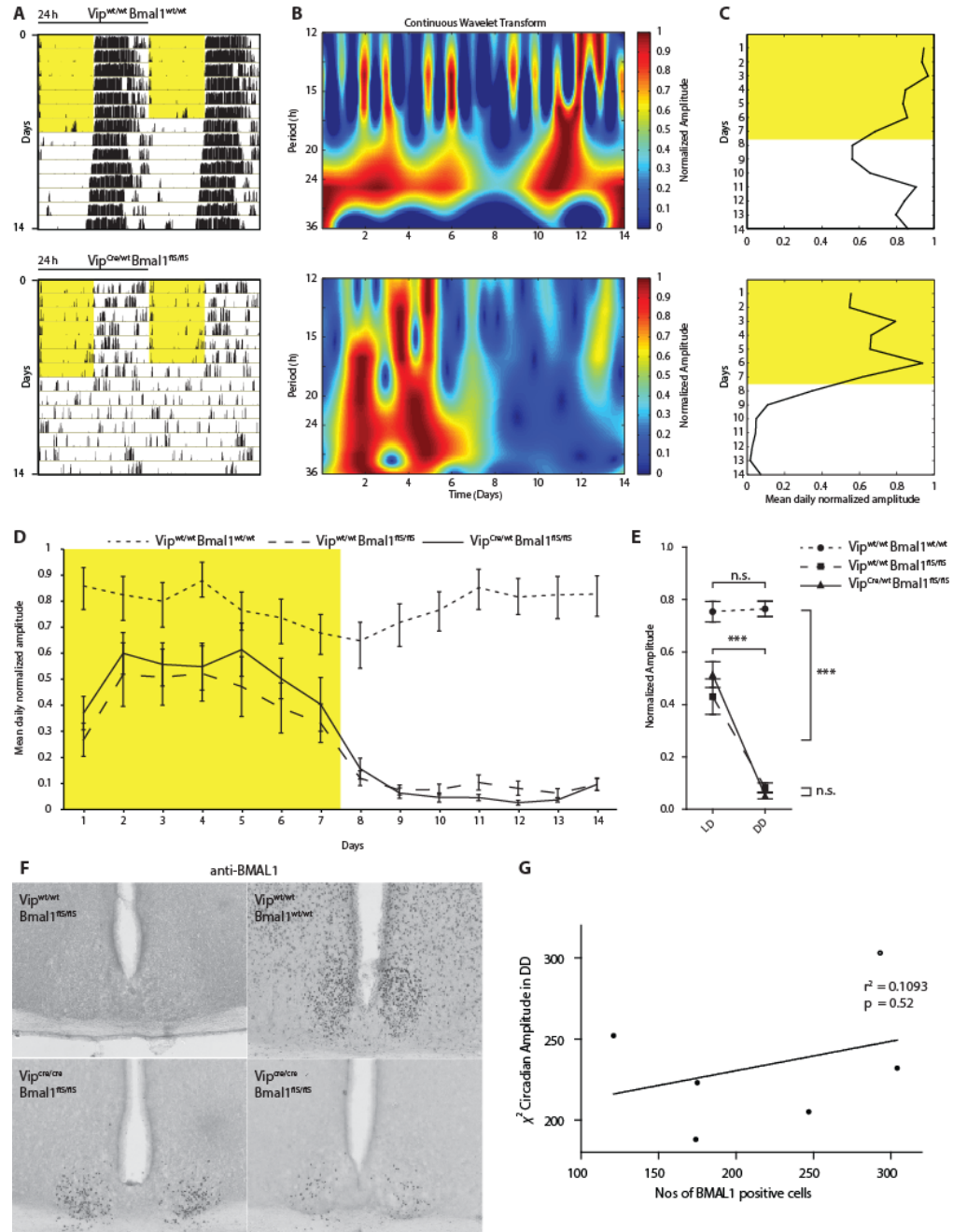


Figure 7: VIP+ subpopulation is not sufficient for behavioural rhythmicity. **(A)** Representative running wheel actograms of WT and VS mice under L:D (Yellow bars) and D:D. **(B)** Dynamics of circadian amplitude revealed by representative continuous wavelet transforms (of mice in A) show a distinct and immediate loss of rhythmicity in VS mice upon release into D:D. **(C)** Mean daily normalized amplitude maximum collapsed from (mice in B) better illustrates the precipitous drop with a strong leftward shift after lights off. **(D)** Summary means of (C) for all genotypes including wildtype (dotted), *Bmal1^{fls/fls}* (dashed), and *Vip^{Cre/wt} Bmal1^{fls/fls}* (solid) mice demonstrates strong circadian amplitude in WT mice but no effect of resurrecting BMAL1 in VIP+ cells. **(E)** Analysis reveals a significant interaction between lighting condition and genotype with post-hoc t-tests revealing no significant effect of lighting condition on WT mice and no significant differences between clock null and clock resurrected mice under any conditions (mean $\pm$ SEM; WT N=8, S N=9, VS N=10; $F_{2,48}=17.65$, Two-way ANOVA, *** $P<0.001$, bonferroni corrected post-hoc t-tests). **(F)** anti-BMAL1 immunohistochemistry images of the suprachiasmatic nucleus (SCN) for *Vip^{wt/wt} Bmal1^{fls/fls}*, *Vip^{wt/wt} Bmal1^{wt/wt}*, *Vip^{Cre/wt} Bmal1^{fls/fls}*, and *Vip^{Cre/wt} Bmal1^{wt/wt}* mice. **(G)** Scatter plot showing the correlation between the number of BMAL1 positive cells and the chi-squared circadian amplitude in DD. The plot shows a weak positive correlation with $r^2 = 0.1093$ and $p = 0.52$.

Chapter 3: A highly-tunable dopaminergic oscillator generates ultradian rhythms of behavioural arousal

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Abstract:

Ultradian (~4 hr) rhythms in locomotor activity that do not depend on the master circadian pacemaker in the suprachiasmatic nucleus have been observed across mammalian species, however, the underlying mechanisms driving these rhythms are unknown. We show that disruption of the dopamine transporter gene lengthens the period of ultradian locomotor rhythms in mice. Period lengthening also results from chemogenetic activation of midbrain dopamine neurons and psychostimulant treatment, while the antipsychotic haloperidol has the opposite effect. We further reveal that striatal dopamine levels fluctuate in synchrony with ultradian activity cycles and that dopaminergic tone strongly predicts ultradian period. Our data indicate that an arousal regulating, dopaminergic ultradian oscillator (DUO) operates in the mammalian brain, which normally cycles in harmony with the circadian clock, but can desynchronize when dopamine tone is elevated, thereby producing aberrant patterns of arousal which are strikingly similar to perturbed sleep-wake cycles comorbid with psychopathology.

Introduction

Ultradian rhythms with periods ranging from one to several hours have been linked to various aspects of mammalian physiology. Usually superimposed on the 24-h diurnal or circadian rhythm, ultradian oscillations have been observed in the context of locomotion, sleep, feeding, body temperature, serum hormones, and brain monoamines in species ranging from fruit flies to humans

(94, 151, 152, 155, 228-233). These physiological cycles most frequently exhibit periods of 2-6 h, adopting harmonics of the 24-h daily light-dark cycle or the endogenous circadian rhythm, when external timing cues are absent. However, the generation of such ultradian rhythms does not depend on a functional circadian system nor a light:dark cycle. Ultradian locomotor oscillations persist in rodents housed in constant darkness even upon ablation of the suprachiasmatic nucleus (SCN), the site of the master circadian pacemaker (94, 231), or genetic disruption of the circadian clock (12, 21) (Figure 8A and B). These ultradian activity cycles may not simply be driven by metabolic demand since the 2 to 3-h rhythm in foraging activity observed in the common vole persists even in the absence of food (234). Studies in this species further indicate that one adaptive value of ultradian activity rhythms may lie in the facilitation of social synchrony, which is suggested to reduce predator risk in this species (235). However, despite their prevalence and hypothesized biological significance, ultradian locomotor rhythms have received little research attention, likely owing to their frequently masked expression and unstable periodicity in contrast with circadian activity rhythms (236, 237) (Figure 8C and D).

In addition to ultradian oscillations, methamphetamine-induced rhythms of locomotor activity occur in the absence of a functional circadian clock. When provided in the drinking water, methamphetamines (Meth) induce a daily activity bout in addition to the expected circadian rhythm (238). This Meth-dependent component – which typically adopts a period in the circadian range – is not abolished upon SCN-lesion nor by genetic disruption of circadian clock function (145, 149). It was thus concluded that a methamphetamine-sensitive circadian oscillator (MASCO) outside the SCN exists which is capable of driving daily cycles of locomotor activity (143). Despite the longstanding recognition of ultradian and Meth-dependent rhythms, the underlying cellular and molecular identity of the oscillator(s) driving them is unknown.

Here we provide evidence for a highly-tunable dopaminergic ultradian oscillator (DUO) which is continuously operative in the mammalian brain and which, together with the circadian clock, orchestrates the daily pattern of arousal. Our data suggest that dopamine acts as both the principal oscillator output as well as an integral component of the DUO, determining oscillator period. Our findings further indicate that the previously described MASCO represents a long-period manifestation of the DUO resulting from elevated dopamine tone. Importantly, our data support an intriguing proposition: that DUO, rather than circadian clock, dysregulation critically contributes to the sleep-wake abnormalities associated with psychopathology.

Results:

Dopamine transporter deficiency results in ultradian locomotor period lengthening

To gain insights into the mechanistic basis of ultradian locomotor rhythm generation, we considered that locomotor activity is associated with an awakened state (239) and consequently, the ultradian locomotor rhythms observed in mice that lack circadian clock function (Figure 1) could be interpreted as rhythms of heightened wakefulness or arousal. In mammals, a key role in arousal promotion has been attributed to distinct monoaminergic neuronal populations located in the upper brainstem and midbrain (240). While altering extracellular levels of the arousal-associated monoamines serotonin, norepinephrine, or histamine by genetic manipulation has only limited effects on locomotion (241-245), depleting the brain of dopamine (DA) profoundly abrogates locomotor activity (246). Moreover, increasing extracellular DA levels induces hyperlocomotion (247) and lengthens the time spent awake (248). We therefore speculated that altering DA tone may affect ultradian rhythm generation. To test this, we examined running wheel activity in mice carrying a disruption in the *Slc6a3* gene, which encodes the dopamine transporter (DAT). *Slc6a3*^{-/-} mice exhibit hyperdopaminergia due to the lack of DAT-mediated DA reuptake into dopaminergic neurons, leading to a hyperactivity phenotype (247, 249). As the presence of the circadian clock and/or a light:dark cycle frequently masks ultradian activity rhythms (237), we assessed the locomotor behavioural consequences of DAT elimination in the absence of the master SCN circadian pacemaker. To do so, we electrolytically lesioned the SCN of *Slc6a3*^{-/-} mice and their wildtype littermates and monitored their running wheel behaviour in constant darkness (DD). While control mice (SCNx-*Slc6a3*^{+/+}) exhibited ultradian activity rhythms with the expected ~4-hr period, SCNx-*Slc6a3*^{-/-} mice showed rhythms whose periods were 3-times longer (Figure 9A and B). Analysis of mice that were deficient for both DAT and the essential clock component BMAL1 (*Bmal1*^{-/-}, *Slc6a3*^{-/-}) corroborated this finding. *Bmal1*^{-/-}, *Slc6a3*^{-/-} mice exhibited ~12-14-h rhythms in locomotor activity, largely phenocopying the SCNx-*Slc6a3*^{-/-} mice, while their *Bmal1*^{-/-}, *Slc6a3*^{+/+} littermates showed ~4-h periods as expected for isodopaminergic mice lacking circadian clock function (Figure 9C and D). Together, these results suggest that DAT removal markedly increases ultradian cycle length. Alternatively, the ~12-h rhythms observed in SCNx-*Slc6a3*^{-/-} and *Bmal1*^{-/-}, *Slc6a3*^{-/-} animals may originate from an independent oscillator, one that is activated by DAT elimination, while the short period ultradian oscillator that operates in DAT intact, SCNx or *Bmal1*^{-/-} animals is disengaged or otherwise obscured.

In order to corroborate that DAT removal indeed lengthens the period of the ultradian activity cycles, we took into account that DAT-mediated DA uptake can be reversed by the selective action of the psychostimulant methamphetamine (Meth)(250). Because Meth leads to increased extracellular DA concentrations as in case of *Slc6a3* gene disruption, we speculated that Meth treatment would result in a, possibly gradual, period lengthening of the ultradian locomotor rhythms. Indeed when we treated *Bmal1*^{-/-} animals with increasing concentrations of Meth via drinking water in DD, we observed a gradual lengthening of the initial ~4-hr locomotor oscillations and this was accompanied by a corresponding increase in activity bout length (Figure 10A and B). Of note, the period increase in response to elevating Meth concentrations did not halt in the circadian range, rather, oscillations continued to lengthen with individual animals reaching periods of 100 hours or more (Figure 10C). Gradual period lengthening of ultradian locomotor rhythms was also observed in *Bmal1*^{-/-} animals exposed to amphetamine (Figure 11A and B), a drug similarly targeting DAT (250), but with lower efficacy than Meth (251). These results argue that DAT targeting psychostimulants affect an endogenous ultradian rhythm generator by increasing period length. However, due to the mode of delivery (drinking water) and the rhythmic Meth uptake that may in turn result from it, it is conceivable that the Meth-dependent, long-period oscillations are “driven” by rhythmic drug intake rather than being generated endogenously. To address this possibility, we subcutaneously implanted *Bmal1*^{-/-} mice with osmotic minipumps that continuously infused Meth over a period of 2 weeks. Running wheel analysis in DD of Meth-infused *Bmal1*^{-/-} animals demonstrated a significant ultradian period lengthening upon drug infusion (Figure 11C and D) suggesting that rhythmic uptake is not required for Meth to exert its period lengthening effect, in line with a previous study performed in rats (149). The relatively limited change in period observed in this experimental paradigm could be due to the short, 2-week infusion timespan, which is perhaps insufficient to robustly lengthen periods beyond 12 hrs (Figure 11D). Together, these findings support the notion that the long-period oscillations observed in Meth-treated animals, and likewise in *Slc6a3*^{-/-} mice, are due to period expansion of an endogenous ultradian rhythm of arousal.

We next aimed to confirm these findings in intact mice. While activity rhythms in the ultradian range are easily discernable in voles and hamsters (252-254), the presence of such rhythms in mice or rats is much less obvious and this has been attributed to masking by the circadian clock (237). Indeed, running wheel activity data from intact mice often do not provide strong indications

of ultradian activity rhythms. However, when we recorded ambulatory behaviour using telemetry, we frequently detected three, evenly spaced activity bouts during the active (night) phase (Figure 12A). The observed ~4-hr peak-to-peak spacing is in line with a previous study on several circadian competent mouse strains where ultradian rhythms with similar period length were detected (151). The absence of a clearly discernable ~4-h rhythmic component during the light portion of the daily LD cycle (Figure 12A) is likely due to masking by the SCN and/or light. Unlike WT mice, *Slc6a3*^{-/-} mice never showed a triple-peak activity pattern at night (Figure 12B) and spectral density analysis in the ultradian (2 to 8-h) range (Figure 12C) underscores that rhythm generation is significantly altered in these animals while total daily activity is not (Figure 12D). When we provided C57BL/6 mice carrying a telemetry implant with Meth in their drinking water, we observed a gradual lengthening of the interval between the night-time activity peaks (Figure 12F and G), resulting in the transformation of the triple peak (Figure 4G, white trace and white triangles) into a dual or single night-time activity peak pattern at the highest (100 mg/l) Meth concentration (Figure 12G, black trace and black triangles). This was corroborated by spectral density analysis revealing a marked reduction in ultradian frequencies upon Meth treatment (Figure 12E). Together, these results indicate that blocking DA reuptake also lengthens ultradian activity bout intervals in circadian competent mice, arguing that an ultradian oscillator is not “activated” by circadian disruption, but rather continuously operative alongside the circadian timer. As with *Bmal1*^{-/-} mice, prolonged Meth-treatment in intact WT mice can lead to profound period lengthening of ultradian rhythms, often into the circadian (48 hr) range (for examples, see Figure 13E, Figure 16F, and Figure 20B).

The antipsychotic haloperidol shortens ultradian locomotor period

Given that both DAT disruption and Meth treatment elevate extracellular DA and concurrently lengthen ultradian rhythm period, we speculated that manipulations aimed at lowering DA tone should conversely lead to period shortening. We therefore provided *Bmal1*^{-/-} mice in DD with the antipsychotic drug haloperidol (Hal) in their drinking water. Hal selectively blocks the DA receptor D2 (DRD2) (255), which is expressed on postsynaptic target sites of DA neuronal projections but also presynaptically as an autoreceptor (256). Importantly, when given chronically as in our case, Hal has been reported to electrically silence midbrain DA neurons (257) and to markedly lower extracellular DA levels in the striatum/nucleus accumbens regions in rats (258, 259). As predicted, *Bmal1*^{-/-} mice responded with successive locomotor period shortening to increasing concentrations of Hal (Figure 13A and B). We also observed Hal-mediated shortening of the long-

period behavioural rhythms in *Bmal1*^{-/-} and wildtype mice treated concurrently with Meth (Figure 13C to F). A period shortening effect of Hal could also be discerned from the core body temperature fluctuations of *Slc6a3*^{-/-} mice, which increased in frequency (Figure 13G). This response, which mirrored the locomotor behavioural response (not shown), was concentration dependent and spectral density analysis confirmed a successive increase of the ultradian component upon Hal exposure (Figure 13H). Together, these data strongly support the hypothesis that both the ultradian rhythms observed in wildtype and *Bmal1*^{-/-} mice and the long-period rhythms previously attributed to the MASCO are driven by the exact same oscillatory mechanism.

As altering extracellular DA is a common denominator of all three manipulations – DAT elimination, (meth)amphetamine, and haloperidol treatment – these findings collectively suggest that DA tone determines ultradian period. Given that DA is known to mediate arousal/wakefulness (240), it appears plausible that DA also serves as principal oscillator output, driving rhythms in arousal. We thus hypothesized that extracellular DA must oscillate in synchrony with the observed activity rhythms.

Extracellular DA fluctuates in synchrony with ultradian activity cycles

To test this hypothesis, *Bmal1*^{-/-} mice were unilaterally implanted with a microdialysis probe positioned along the ventro-dorsal extent of the right striatum (Figure 14G), a site heavily innervated by DA neurons. Mice were kept under constant dim red light (<5lux) and ultradian activity rhythms were monitored using infra-red beam breaking. The animals showed the expected short period rhythms in locomotor activity throughout the experiment (Figure 6A), and analysis of dialysates revealed that extracellular DA levels fluctuated concordantly with the activity cycles (Figure 14A and Figure 15A, compare solid red and black traces). Importantly, we generated a model oscillation using the cosinor method from locomotor rhythms recorded for 20 hrs after dialysate sampling with no investigator present, and compared it to the DA fluctuations observed. The goodness of fit between the cosinor and the DA profiles indicated by the sum of squared errors (SSE=0.050 ± 0.027, mean ± SEM, N=7) suggests that the dialysate sampling procedure itself did not perturb the generation of the endogenous ultradian activity cycles. To determine the statistical significance of the observed agreement between the cosinor and the DA trace, we randomly permuted the individual time points of the DA trace and determined the percentage of permuted traces with an equal or better fit to the cosinor in comparison to the observed DA fluctuation. On average, only 3.2 ± 1.7% of

100,000 permuted DA profiles correlated as well or better than the experimentally observed DA measurements (Figure 14B and Figure 15B), confirming that extracellular DA fluctuates in synchrony with the ultradian activity cycles. Of particular note, linear regression revealed a highly significant correlation between mean DA concentration in the dialysate and ultradian locomotor period in the *Bmal1*^{-/-} animals we tested (Figure 14C), which again supports a role of (extracellular) DA as a period determinant of the ultradian activity cycle.

As our findings suggest that the observed >24-h activity rhythms are due to period lengthening of the ultradian oscillations, we similarly expected DA levels to fluctuate in synchrony with the activity cycles in Meth treated animals. To test this, we measured DA content in tissue punches of Meth-treated *Bmal1*^{-/-} mice from the dorsal (caudate putamen, CPu) and ventral (nucleus accumbens, NAcc) striatum as well as the ventral midbrain, which includes both the substantia nigra (SN) and the ventral tegmental area (VTA) (Figure 14G). For punch collection, animals were sacrificed during their active and inactive phases, respectively (Figure 6F). We detected significantly higher levels of DA as well as its immediate metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), in tissue extracts of the CPu during the animal's active phase (Figure 14D). There was also a significant increase in DOPAC levels and a trend towards elevated DA ($P=0.066$) during the active period in the NAcc, while extracts from the midbrain region, which contains the cell bodies of the striatal dopaminergic afferents, exhibited no detectable change (Figure 14D).

We also found a significant correlation between locomotor period and DA content in CPu and midbrain punches collected during the active phase (Figure 14E), again consistent with a role for DA as a period determinant of ultradian oscillations.

*SCN-intact *Slc6a3*^{-/-} mice show two components of rhythmic activity*

As *Slc6a3*^{-/-} mice lack the triple peak night-time activity pattern that is characteristic of wildtype mice, we subjected *Slc6a3*^{-/-} mice to long-term running wheel activity monitoring to examine their locomotor behaviour in more detail. Under LD conditions, we observed more fragmented daily activity in *Slc6a3*^{-/-} than their wildtype counterparts (Figure 16A and D). Upon release into constant darkness (DD), wildtype animals exhibited a daily rhythm with a period slightly shorter than 24 hrs as expected for mice (21) (Figure 16A). While this principal locomotor component was also observed in *Slc6a3*^{-/-} mice, they repeatedly exhibited an additional activity component with periods longer than 24 hrs (Figure 16D to F). This component, which was more evident after the first few

weeks in DD, extended from the main activity component and lasted for several cycles before disappearing late in the subjective day (Figure 16D). Overall, there was no significant difference in the total amount of daily activity even though the temporal pattern of locomotion in these mice is severely altered (Figure 16C). The observed dual component activity pattern shows striking similarities to the activity patterns of Meth-treated C57BL/6 mice (Figure 16G to I), suggesting that the second rhythmic component is generated by the dopaminergic oscillator.

However, such locomotor activity pattern, with two components oscillating at different frequencies, has also been observed in rats housed under a 22-h LD cycle and have been attributed to a dissociation of molecular rhythms between the dorsal and ventral SCN (179). To rule out that the SCN is similarly affected in *Slc6a3*^{-/-} mice, we sacrificed mice at the peak and trough of the main activity component when the second rhythm was maximally antiphasic (Figure 17A). In situ hybridization directed against *Period1* transcripts did not indicate rhythmic desynchrony between or within SCN hemispheres. The riboprobe showed strong, homogenous staining throughout the SCN in the early subjective day, whereas little to no signal was detectable in the early subjective night, suggesting that SCN pacemaker function is unperturbed in *Slc6a3*^{-/-} mice (Figure 17B).

Together, these findings thus argue that the second rhythmic component of *Slc6a3*^{-/-} mice is indeed a product of the above described dopaminergic oscillator.

Chemogenetic activation of DA neurons lengthens ultradian period

Since striatal and midbrain DA levels vary concordantly with activity rhythms in *Bmal1*^{-/-} mice, and DA neurons of the SN/VTA are by and large the exclusive source of striatal and midbrain DA, these neurons are a likely site of ultradian rhythm regulation, if not generation. To corroborate the importance of these neurons in the oscillator process, we employed a chemogenetic approach based upon DREADD (designer receptors exclusively activated by designer drugs) technology (260). We stereotactically delivered the adeno-associated virus *AAV-DIO-hM3Dq-mCherry* (260) into the SN/VTA region of mice that carried an *Slc6a3* promoter driven *Cre* transgene (*DAT-Cre*) (Figure 18A)(261) and that additionally were either BMAL1-deficient (*DAT-Cre*, *Bmal1*^{-/-}) or received an SCN lesion (*DAT-Cre*, SCN_x). Upon Cre-mediated recombination, virally transfected cells express the stimulatory DREADD hM3Dq, which has been demonstrated to increase neuronal firing frequency upon binding of the compound clozapine-N-oxide (CNO), which is otherwise physiologically inert (262). As augmenting firing frequency in DA neurons enhances DA release

(263), and CNO stimulation of successfully transduced *DAT-Cre* mice evokes vigorous firing of dopamine neurons (264), CNO is expected to increase DA release. Consistently, i.p.-injection of CNO elevated extracellular DA in the striatum of those mice and led to a prolonged increase in locomotor activity (Figure 18C and D), which was absent in vehicle injected animals (Figure 8-figure supplement D). Responsive mice showed mCherry fusion tag expression selectively in tyrosine hydroxylase (TH) positive cells (Figure 18B, Figure 19A). Cell counting revealed that, $92.9 \pm 5.1\%$ and $87.8 \pm 6.0\%$ (mean $\pm$ SEM, N=6 for both groups) of the midbrain TH⁺ neurons also expressed the mCherry fusion tag in responsive *DAT-Cre*, *SCNX* and *DAT-Cre*, *Bmal1*^{-/-} mice, respectively, confirming effective Cre-mediated hM3Dq expression in midbrain DA neurons. Running wheel activity monitoring of virus-injected mice showed the expected short period locomotor oscillations, however, upon switching to CNO-containing drinking water (red arrows), both, *AAV-hM3Dq* transduced *DAT-Cre*, *SCNX* and *DAT-Cre*, *Bmal1*^{-/-} mice responded with locomotor period lengthening (Figure 18E-H), an effect that was reversed when mice were returned to pure water (Figure 18G, blue arrows). As expected, a period lengthening was not observable in *AAV-hM3Dq* injected *DAT-Cre*^{0/0}, *Bmal1*^{-/-} mice upon CNO exposure (Figure 19, B and C). These results further corroborate that elevating DA tone, in this case by selective excitation of DA neurons, lengthens ultradian locomotor period and that the oscillator driving ultradian rhythmicity comprises DA neurons of the VTA/SN region.

Discussion:

Collectively, our results provide strong evidence that a dopaminergic ultradian oscillator (DUO) driving rhythms of behavioural arousal is continuously operative in the mammalian brain. We propose that under normal conditions, this DUO cycles in harmony with the circadian SCN pacemaker and that the rhythmic information of both the SCN and the DUO are integrated at a common downstream site to create the daily pattern in locomotor activity (Figure 20A). However, elevation of DA tone can lead to DUO period lengthening, which either results in DUO free-run (for example, Figure 16D and G) or reinstatement of oscillator synchrony albeit at a different harmonic (Figure 20B, e.g. 48 hrs). The DUO appears as a highly-tunable oscillator, able to adopt period lengths from a few hours to multiple days. This is in stark contrast to the circadian timer which cannot adopt periods that are more than a few hours off from 24 hrs when its limits of entrainment are tested experimentally (265).

Given that DA is known to stimulate locomotor activity (246), our observation of a cyclical rise in extracellular striatal DA, which is in synchrony with ultradian activity rhythms, argues that DA acts as an output of the DUO. Our finding that manipulations affecting extracellular DA levels alter oscillator period and that extracellular DA tone shows a remarkably high correlation with activity cycle length, strongly suggests that DA is a period determinant and therefore must be an integral component of the oscillatory mechanism itself. As all period-altering manipulations directly impinge upon DA neuronal physiology this suggests that either i) DA neurons are the site of ultradian rhythm generation or ii) they are a key cog in the oscillatory mechanism. The chemogenetic activation experiments indicate that the relevant DA neuronal population is located in the midbrain as selective activation of DA neurons in this region had a period lengthening effect on the ultradian activity. Future experiments will be aimed at delineating the precise DA population(s) required for the ultradian rhythm generation process and how rhythmic synchrony between neurons is maintained, if the DUO is indeed composed of a population of cellular oscillators.

Our data indicate that the circadian and ultradian locomotor rhythms are normally harmonized (for instance, Figure 20A), suggesting that the circadian pacemaker and the DUO interact. Of note, extracellular DA was reported to fluctuate diurnally in the rodent striatum (266), which was abrogated in *Slc6a3*^{-/-} mice (159). In these studies, only group averaged DA profiles were presented and DA was solely measured in circadian intact mice and rats, thus it is conceivable that any ultradian component (in WT) or infradian component (in *Slc6a3*^{-/-}) escaped detection. Critically, the observation that the DA levels, on average, followed a diurnal pattern with a night-time peak, together with the observation that ultradian locomotor period in female hamsters is longer in the dark versus the daily light phase (254), is consistent with the LD cycle and/or the circadian pacemaker affecting DUO rhythmicity by altering (extracellular) DA. Note however, that dopamine content in whole brain extracts from circadian-intact rats kept under LD showed a strong ultradian variation with no obvious evidence for a diurnal rhythmic component (267). Also, microdialysis did not reveal a day:night difference in extracellular DA levels in the striatal NAcc region when measured in DD (268). This same study reported elevated DA levels and hyperactivity in mice lacking clock gene *Rev-erba*, suggesting that the circadian clock, possibly intrinsic to DA neurons themselves, has a role in DA regulation. Indeed, knockdown of the core circadian clock component *Clock* in DA neurons of the murine VTA, increases electrical firing rate in VTA neurons and enhances locomotor response to novel objects (269). Interestingly, we did not observe any

systematic differences in ultradian locomotor period between SCN_x and *Bmal1*^{-/-} mice in constant darkness conditions, regardless of DAT status (Figure 8 and Figure 9), suggesting that extra-SCN circadian clocks have no role in DUO-mediated ultradian locomotor rhythm generation.

Our data also provides evidence that the postulated MASCO (143) represents a long-period manifestation of the DUO as a result of methamphetamine's action on the dopamine transporter, blocking dopamine reuptake thereby increasing extracellular DA levels. Interestingly, the dopamine system has been also implicated with another behavioural timing system: the food-entrainable oscillator (FEO). This circadian independent oscillator (270-272) drives food-anticipatory locomotor activity (FAA) that emerges when food access is restricted to a few hours each day (273). D1 (DRD1) and D2 (DRD2) receptor antagonists attenuate FAA additively (274) while pharmacological DRD2, but not DRD1, activation altered the phase of FAA (161). Most recently, using knockout mice, it was revealed that DRD1 but not DRD2 is necessary for the appropriate expression of FAA and that rescuing dopamine signalling selectively within the dorsal striatum was sufficient to restore FAA in dopamine deficient mice (159). Given the links to the dopaminergic system, it will be of interest to investigate whether the DUO has a role in the temporal regulation of FAA.

While these findings argue that food cues engage the dopamine system to alter daily locomotor activity patterns, it is clear that dopamine signalling also affects food intake as mice lacking dopamine are lethargic and do not actively consume food (28). Considering that the DUO is a universal driver of ultradian behavioural rhythms in mammals, the finding that ultradian bouts of running-wheel and feeding activity are co-expressed in the common vole (35) suggests that dopamine can synchronously drive food seeking and general activity, which is in line with the view that dopamine acts as a general promoter of motivated arousal.

Slk6a3^{-/-} mice have been proposed as a model for schizophrenia (275) and the DA hypothesis of schizophrenia states that DA elevation is causal to the behavioural symptoms of this psychiatric condition. Intriguingly, circadian (48 hr) or free-running rhythms in locomotor activity reminiscent of the behavioural patterns we detected in *Slk6a3*^{-/-} or Meth-treated mice (Figure 12F, Figure 16C and F, Figure 20B) have been observed in schizophrenic subjects (276, 277), suggesting that DUO dysregulation underlies the rest-activity aberrations associated with schizophrenia. Furthermore, actigraphy recordings in schizophrenic patients also revealed that Hal treatment reduces circadian/diurnal locomotor amplitude and leads to the emergence of ultradian activity bouts (278,

279), effects we likewise observed in *Slc6a3*^{-/-} mice in response to Hal. Transient DUO period lengthening might equally account for the rest-activity pattern abnormalities observed during manic episodes in bipolar disorder, which has been also associated with altered DA tone (280). Bipolar subjects have been reported to show rapid, 48-h cycling between mania and depression (281, 282), one to multiple 48-h sleep-wake cycles when switching from depression to mania (283), or a long-period ‘free-running’ rhythm of wakefulness (284). Thus schizophrenic and bipolar subjects both appear to exhibit rest-activity cycle aberrations strikingly similar to those observed in *Slc6a3*^{-/-} or Meth-treated mice, suggesting that DUO dysregulation is a common indicator for these psychopathologies and perhaps even a common disease cause.

A switch to sleep-wake cycles with a period much longer than 24 hrs have also been observed in subjects that were studied in temporal isolation (8). Because other physiological parameters such as urine secretion and core body temperature showed phase-aligned, circadian fluctuations with a period much closer to 24 h, the subjects were considered internally desynchronized. Notably, affected subjects tend to exhibit high scores of neuroticism (285). It is therefore conceivable that the observed internal desynchronization is also due to a dysregulation of the DUO.

Materials and Methods:

Animals *Bmal1*^{-/-} (21) and DAT-Cre (261) mice were on a C57BL/6J genetic background, while *Slc6a3*^{-/-} mice (247) were maintained on a mixed C57BL/6JxDBA/2J background (286). Animals were housed under LD 12hr:12hr unless otherwise stated. *Slc6a3*^{-/-} mice were found to exhibit high attrition rates (~80%) in experiments that involve long-term locomotor activity monitoring. To increase survival, *Slc6a3*^{-/-} mice were first group-housed for 1 week after transfer into the light-tight, ventilated cabinets used for activity monitoring. This was followed by 1 week of individual housing in running wheel cages with the wheels locked before commencing baseline activity recordings. During this adaptation phase and throughout the subsequent experimental period, both *Slc6a3*^{-/-} mice and their wildtype littermates were provided with ad libitum chocolate flavored chow (Supreme Mini-Treats, BioServ) as well as cotton nestlets and ample shredded corrugated card stock. This regimen significantly reduced attrition rates to less than 20% on average. Experimental procedures were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the McGill University Animal Care Committee.

Locomotor activity and core body temperature monitoring *Running wheels:* Animals were individually housed in light-controlled cabinets and activity was recorded continuously (ClockLab, Actimetrics). Actograms, displaying binned running wheel revolutions per 6 minutes (0.1 hr), and the associated Lomb-Scargle periodograms, displaying amplitude, were generated using ClockLab software. *Telemetry:* Animals were individually housed in standard cages placed atop energizer/receiver units (ER-4000, Mini Mitter). One week prior to data collection, electromagnetic induction powered telemetry probes (G2 E-mitter, Mini Mitter) were implanted intraperitoneally. Locomotion, measured in counts per minute, and core body temperature (BT, in °C), was collected in 6-min bins (0.1 hr) using Vitalview software (Mini Mitter). BT data was exported into Clocklab to generate actogram-style data displays with tick mark height corresponding to temperatures from 34–38°C; For waveform generation, averaging, and 2-hr recursive smoothing, locomotor activity and BT data was exported to Excel (Microsoft). Matlab (Mathworks) was used for ribbon plot generation, and low pass Butterworth filtering (1 hr).

Pharmacology Stock solutions of (+)-Methamphetamine hydrochloride (100mg/l, Sigma), D-Amphetamine hemisulfate (100mg/l, Sigma), Haloperidol (11.25 mg/ml, Sigma) and Clozapine-N-Oxide (15mg/l, NIMH) were prepared using tap water. Methamphetamine solutions were adjusted to pH 7 using sodium hydroxide and haloperidol was dissolved by stirring at 40°C.

Chronic methamphetamine infusion Mice were continuously infused with methamphetamine (0.6mg/day in 0.9% saline) for 14 days using subcutaneously implanted osmotic minipumps (Alzet, Model 1002). Pumps were fitted with a 65-mm polyvinyl chloride catheter (Plastics One, 0.69-mm inner diameter) and backfilled with saline to allow for an approximately 4 day post-surgery recovery prior to drug exposure.

In-situ hybridization In situ hybridization was performed as previously described (121). Briefly, following decapitation, brains were removed and quickly frozen at –80°C. Serial coronal hypothalamic brain slices (25µm) were collected using a cryostat and stored at –80°C until hybridization. Sections were hybridized overnight at 60°C to a digoxigenin-labelled riboprobe targeting the coding regions of mouse *Per1* (nucleotides 579-1478, Genbank, NM_011065.4).

SCN lesions Electrolytic lesions of the suprachiasmatic nucleus were performed as described (199). Briefly, an electrode (RNE-300X, Rhodes Medical Instruments) was lowered through a hole drilled in the skull at the mid-sagittal sinus according to stereotaxic coordinates (AP -0.25 mm, DV -6.00

mm from Bregma) and a constant current (2mA, 10s; D.C. Constant Lesion Maker, Grass Instruments) was applied. Only mice with behavioural circadian arrhythmia (in the 20 to 28-hr range assessed by Lomb-Scargle periodogram analysis) and subsequent post-mortem histological verification using DAPI staining mounting medium (Vectashield, Vector Labs) were included for analysis. This represented approximately 50% of lesioned mice when averaged across all studies.

In vivo microdialysis One week prior to sampling, mice were stereotactically implanted with a guide cannula (C312G/spc 2.5 mm below pedestal, Plastics One), targeting the striatum (AP +1.10 mm, DV +5.5 mm, ML +0.9 mm). One day prior to dialysis, mice were transferred to a beam break monitor (VersaMax, Accuscan Instruments) and tethered using a dummy probe assembly under constant, dim, red light (<5lux). On the day of sampling, in-house recording probes (287), were connected to a central swivel (Model 72-0000, Harvard Apparatus). Artificial cerebrospinal fluid was delivered via syringe pump (Model 403, CMA Microdialysis) at a flow rate of 1.0 μ l/min. This procedure was similarly followed to test the effects of CNO in AAV-transduced DAT-Cre mice where intraperitoneal injections of 1mg/kg CNO were given after approx. 1h of baseline sampling. Samples were collected every 20 min for 4 hrs and immediately admixed with 1 μ l of perchloric acid. DA and DOPAC content in the dialysate were determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC) as described (288). Chromatographic peak analysis was conducted using CoulArray software (ESA Inc.). After data collection, brains were removed, sectioned and stained with hematoxylin to verify probe placement. Locomotor activity data, recorded as the number of beam breaks per minute, were exported into Matlab (Mathworks) for waveform generation, 20-min binning, cosinor modelling, and false discovery rate analysis.

DA/DOPAC content determination in tissue extracts After decapitation, brains were quickly removed and frozen (-80°C). 320 μ m coronal brain slices were obtained by cryosectioning and then microdissected (1- or 2-mm diameter sample corers, Fine Science Tools Inc.) to obtain tissue of the CPu (2 mm), NAcc (1 mm), and SN/VTa midbrain (2mm) regions (see Figure 6G for punch location). DA/DOPAC was quantified as previously described (288). Briefly, individual punches from each region were homogenized in 45 μ l perchloric acid (0.25 M) to which 15 μ l of a 100 ng/ml solution of 3,4-dihydroxybenzylamine was added, which served as the internal standard. Concentrations were determined by HPLC-EC. After perchloric acid extraction, the protein containing pellets were reconstituted in 0.1 N sodium hydroxide for protein quantification (Pierce BCA Kit, Thermo Scientific)

Chemogenetics Mice were anaesthetized with isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments). Recombinant AAV8-DIO-h3MDq-mCherry (260)(titer = 3×10^{12} genomes copies per ml, UNC Vector Core Services, Chapel Hill) was bilaterally injected into the VTA/SN area (coordinates from bregma: AP: -3.44 mm, DV: -4.40 mm, L: ± 0.48 mm) (289) through a cannula (33 gauge, Plastics One) at a flow rate of 0.05 μ l/min for 10 min (0.5 μ l total volume per side) using a syringe pump (Harvard Apparatus). Mice were subsequently maintained in individual housing for at least 2 weeks prior to CNO treatment.

Immunohistochemistry Immunostaining was performed as previously described (225) using cryoprotected (30% Sucrose), free-floating coronal sections from fixed brains collected after intracardiac perfusion (4% paraformaldehyde) and cut at 40 microns using a cryostat (Leica). For fluorescent labeling, sections were incubated overnight with primary antibodies for mCherry (rabbit anti-RFP, 1:1000, Rockland) to enhance detection of the mCherry expression and antibodies for tyrosine hydroxylase (mouse anti-TH, 1:1000, Millipore). This was followed by 2h incubation with secondary antibodies (Life technologies, anti-rabbit Alexa Fluor 567 and anti-mouse Alexa Fluor 647, 1:250) after which sections were mounted on superfrost slides (VWR), coverslipped with Vectashield mounting medium (Vector Labs) and imaged by fluorescence microscopy (AxioObserver Z1, Zeiss). Quantification of co-expression was performed on a single, medial, midbrain section from each behaviourally responsive mouse using the cell counter plugin of ImageJ (National Institute of Health) in order to determine the percentage of all TH-positive cells that also expressed the hM3Dq--mCherry fusion protein (N=6 per genotype).

Statistical analysis One-way and repeated measures ANOVA, t-tests, and linear regression analyses were performed using Prism 5 (GraphPad). Planned comparisons for the repeated measures were carried out to determine significant period changes between subsequent measurements. Bonferroni correction was used for post-hoc testing of individual group differences.

False discovery rate (FDR) and Cosinor determination To evaluate the probabilistic significance of the least-square fit between the DA trace and the cosinor model that was computed based on the animal's locomotor activity oscillations, a false discovery rate approach was employed (199). For cosinor computation, we first determined the locomotor period of *Bmal1*^{-/-} mice by Lomb-Scargle periodogram analysis of 20 hrs of locomotor recordings following dialysate sampling. The determined period was then used as an input parameter for a least-squares cosinor analysis (290)

using a custom script and function written for Matlab (<https://github.com/storchlab/Cosinor-FDR.git>). This procedure generated a best-fit (co)sine wave modelling the activity time-series. This model wave was then used to assess the concordance between the extracellular DA fluctuations and locomotor oscillations. To calculate FDR, we randomly permuted the temporal order of the measured DA concentrations 100,000 times, and assessed its fit to the cosinor. The permutation procedure will degrade the fit of any truly rhythmic signal but will have little to no effect on noisy or flat profiles. The probability of false discovery was calculated as the percentage of randomly permuted traces that show an equal or better fit to the cosinor than the measured DA fluctuation.

Butterworth filtering Low pass filtering (1-h cut-off) of raw data was conducted using a Butterworth zero-phase filter (291) in Matlab. This allowed for better visualization of ultradian frequencies in the >1-h range, in a manner similar to recursive smoothing but without phase distortion.

Period determination Ultradian rhythms show variability in amplitude and period. We thus used Lomb-Scargle periodogram analysis (Clocklab) to estimate ultradian period length as this method is relatively tolerant to noisy data and data gaps, which can result from intermittent ultradian rhythm expression (292). Unless otherwise stated, period length was determined by identifying the highest peak above the significance threshold ($\alpha=0.01$) in the Lomb-Scargle periodogram. Subsequent plotting of an actogram at the determined modulus was conducted in each case in order to visually confirm rhythmicity and rule out the false identification of harmonic frequencies or side-lobes due to leakage (293).

Continuous wavelet transforms To visualize and quantify the dynamics of period and amplitude in circadian incompetent mice, continuous wavelet transforms using the Morlet wavelet (222) were performed for the 1 to 12-h range using custom algorithms written in Matlab (<https://github.com/storchlab/CWT.git>). Locomotor activity data of seven consecutive days per individual SCN-lesioned or *Bmal1*^{-/-} mouse was used. For each spectrogram, a ridge identifying the peak amplitude of the spectrum was generated as described (223) and the associated values were used to calculate the average and standard deviation of the dominant ultradian frequency for each animal.

Amplitude spectral density (ASD) To determine the prevalence of oscillations in a given period range we calculated the area under the curve of all significantly rhythmic periodicities ($\alpha=0.01$) in

the Lomb-Scargle periodogram, i.e. the spectral density. For normalization, the obtained spectral density was divided by the total significant spectral density in the 0 to 40-h range and expressed as percentage.

Acknowledgments:

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Figures:

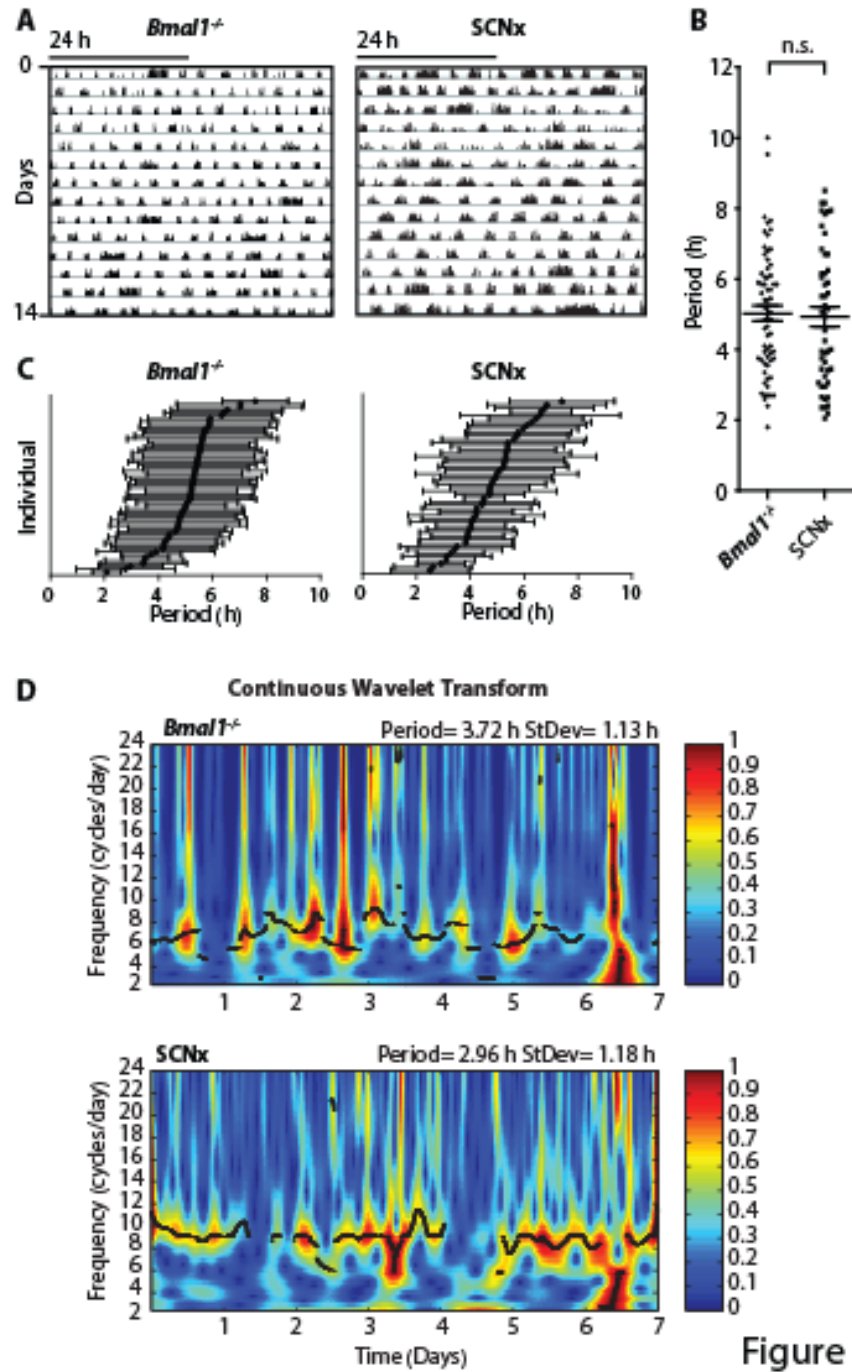


Figure 8: Inter- and Intra-animal variability of ultradian activity rhythms across time in circadian incompetent mice. **(A)** Representative double-plotted actograms of running wheel activity in SCN-lesioned (SCNx) and *Bmal1*^{-/-} mice in DD. **(B)** Dot plot of locomotor period length in DD based on Lomb-Scargle periodogram analysis of 7 consecutive days of activity recording (N=65 for *Bmal1*^{-/-} and N=48 for SCNx; $t_{111}=0.2785$, $P=0.78$, unpaired t-test). **(C)** Intra-animal period variability expressed as mean $\pm$ SD for each animal, ranked according to mean period length derived from continuous wavelet transforms (CWT) for the 1 to 12-h frequency range (same animals and timespans for calculation as in b). **(D)** CWT-heatmaps showing decibel scaled and normalized amplitude of oscillations according to frequency and time with black traces indicating the ridge of local amplitude maxima.

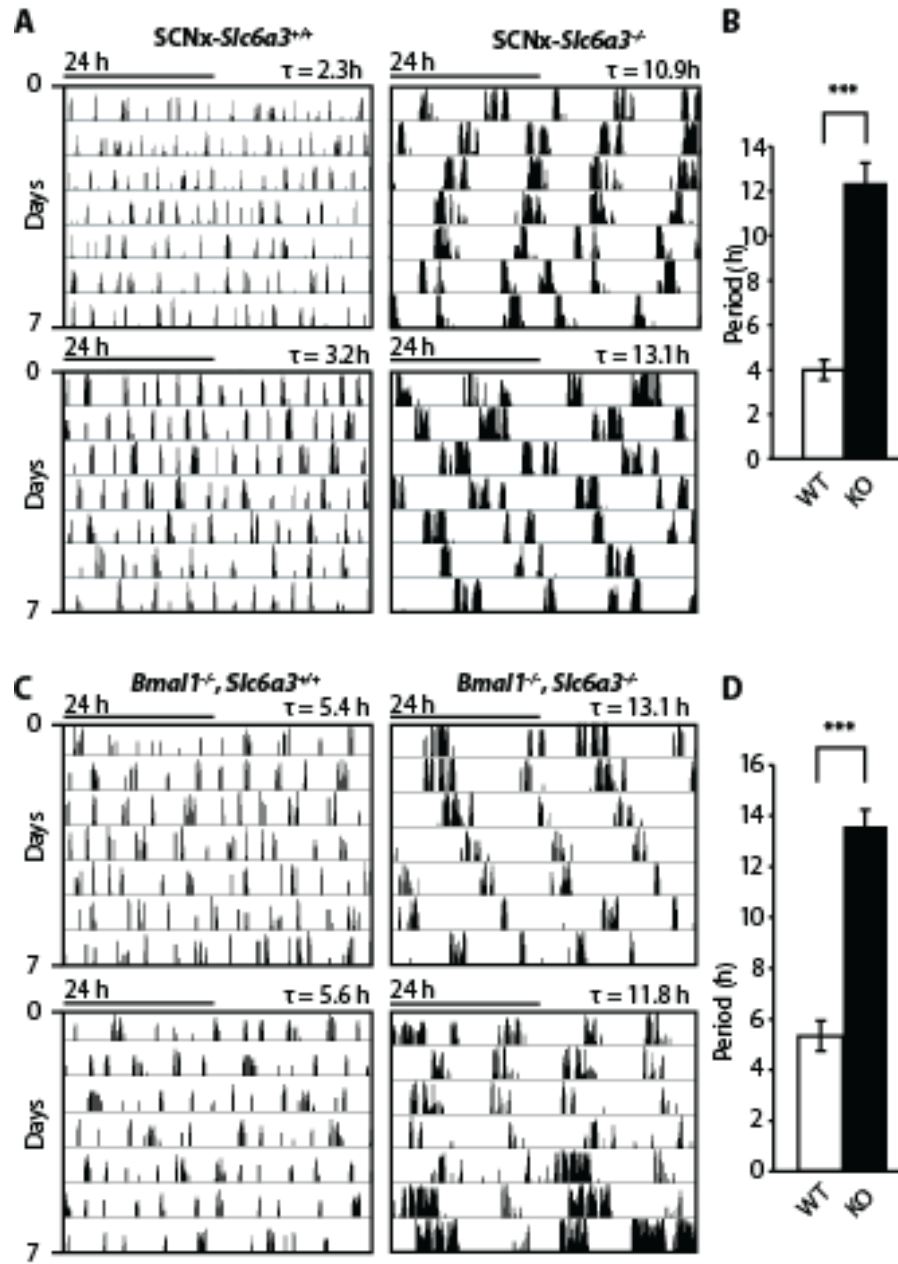


Figure 2

Figure 9: Dopamine transporter knockout alters periodicity of ultradian locomotor rhythms in circadian incompetent mice under constant darkness. **(A)** Representative, double-plotted actograms demonstrating marked lengthening of ultradian locomotor periods in *SCNx-Slc6a3*^{-/-} mice as compared to *SCNx-Slc6a3*^{+/+} littermates. Tau(τ) indicates individual period. **(B)** Period length averages of ultradian activity (N=7; $F_{1,6}=253.8$, *** $P<0.0001$, ANOVA) from Lomb-Scargle periodogram analysis of 7-day time-spans. **(C)** Representative, double-plotted actograms demonstrating markedly increased ultradian period lengths in *Bmal1*^{-/-}, *Slc6a3*^{-/-} mice as compared to *Bmal1*^{-/-}, *Slc6a3*^{+/+} mice. **(D)** Period length averages of ultradian activity (N=4; $F_{1,3}=194.2$, *** $P<0.001$, ANOVA) from Lomb-Scargle periodogram analysis of 7-day time-spans.

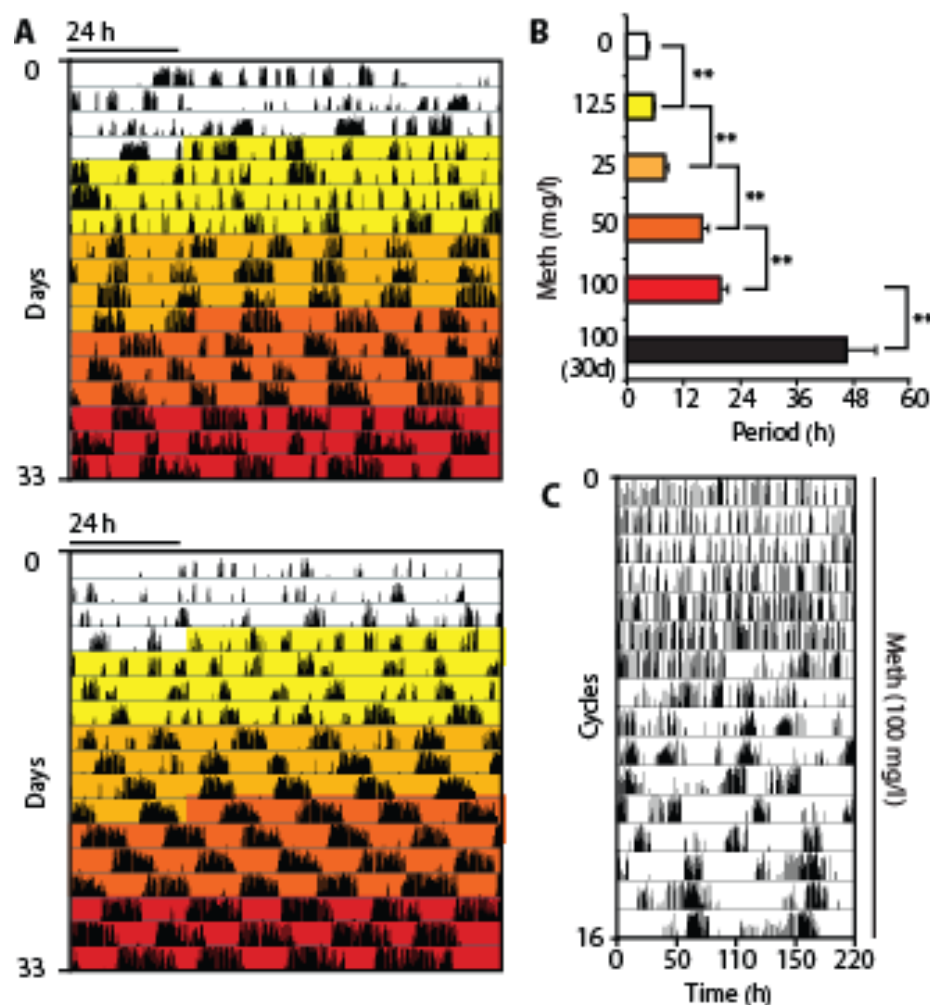


Figure 3

Figure 10: Pharmacological tuning of DAT activity by incrementally increasing methamphetamine concentrations lengthens ultradian locomotor period into the infradian range (A) Representative actograms of Meth-treated *Bmal1*^{-/-} mice in DD. Treatment intervals are highlighted with corresponding concentrations indicated in (B). (B) Mean periods from the last seven days at a given Meth concentration. Repeated measures ANOVA revealed a significant main effect of concentration/time ($F_{5,40}=34.30$, $P<0.001$) and significant period lengthening between consecutive concentrations (mean $\pm$ SEM; N=12; $**P\leq 0.005$, planned comparisons; 30d, 30 day exposure to 100mg/l). (C) Modulo 110-h actogram of a *Bmal1*^{-/-} animal after extended exposure to Meth revealing an ultra-long activity rhythm.

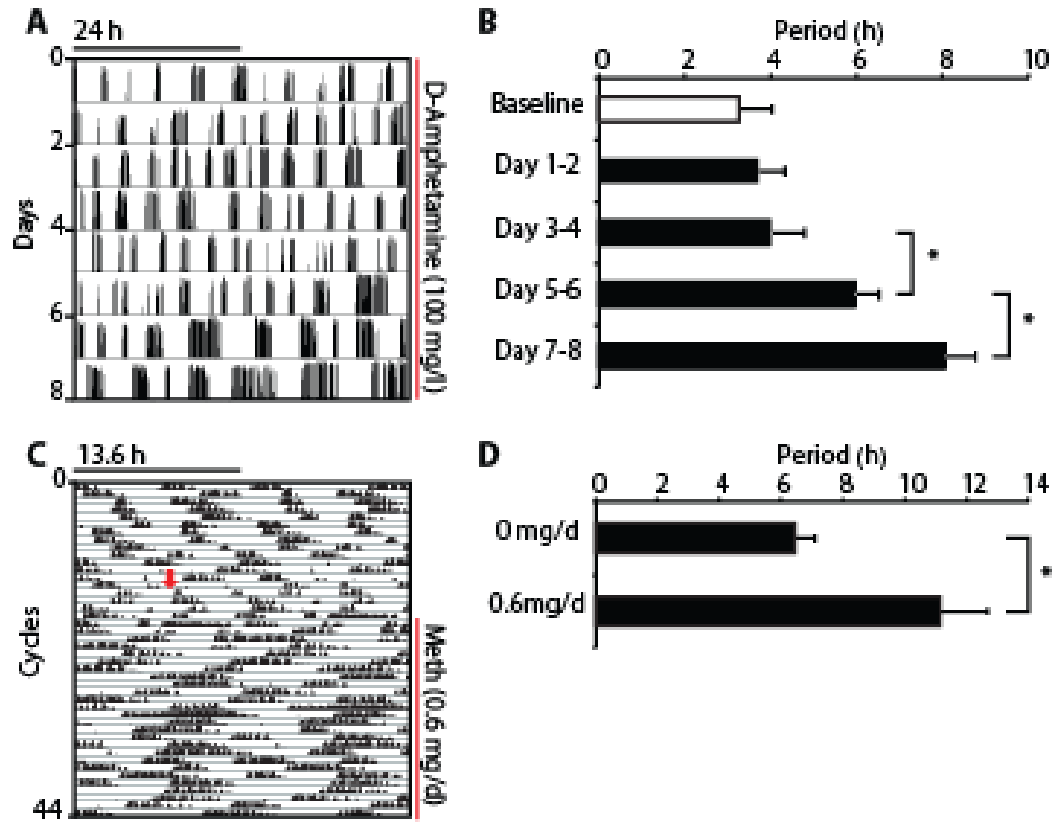


Figure 11: Amphetamine treatment lengthens ultradian locomotor rhythms in *Bmal1*^{-/-} mice. **(A)** Representative actogram of *Bmal1*^{-/-} mice in DD provided with d-amphetamine (100mg/l) in their drinking water. **(B)** Average activity rhythm periods calculated for the indicated days post treatment-onset (mean $\pm$ SEM, N=10; $F_{4,9}$ =26.51, P <0.0001, repeated measures ANOVA; * P ≤0.001, planned comparison). **(C)** Representative running wheel activity actogram (plotted modulo 13.6 hrs) of *Bmal1*^{-/-} mice in DD, which received chronic subcutaneous infusions of Meth (0.6mg/day) for 14 days. Red arrow indicates time of pump implantation. **(D)** Average activity rhythm periods during the last 7 days prior to pump implantation (0mg/day) and the last 7 days of treatment (mean $\pm$ SEM, N=6; $F_{1,5}$ =3.525, * P <0.01, ANOVA).

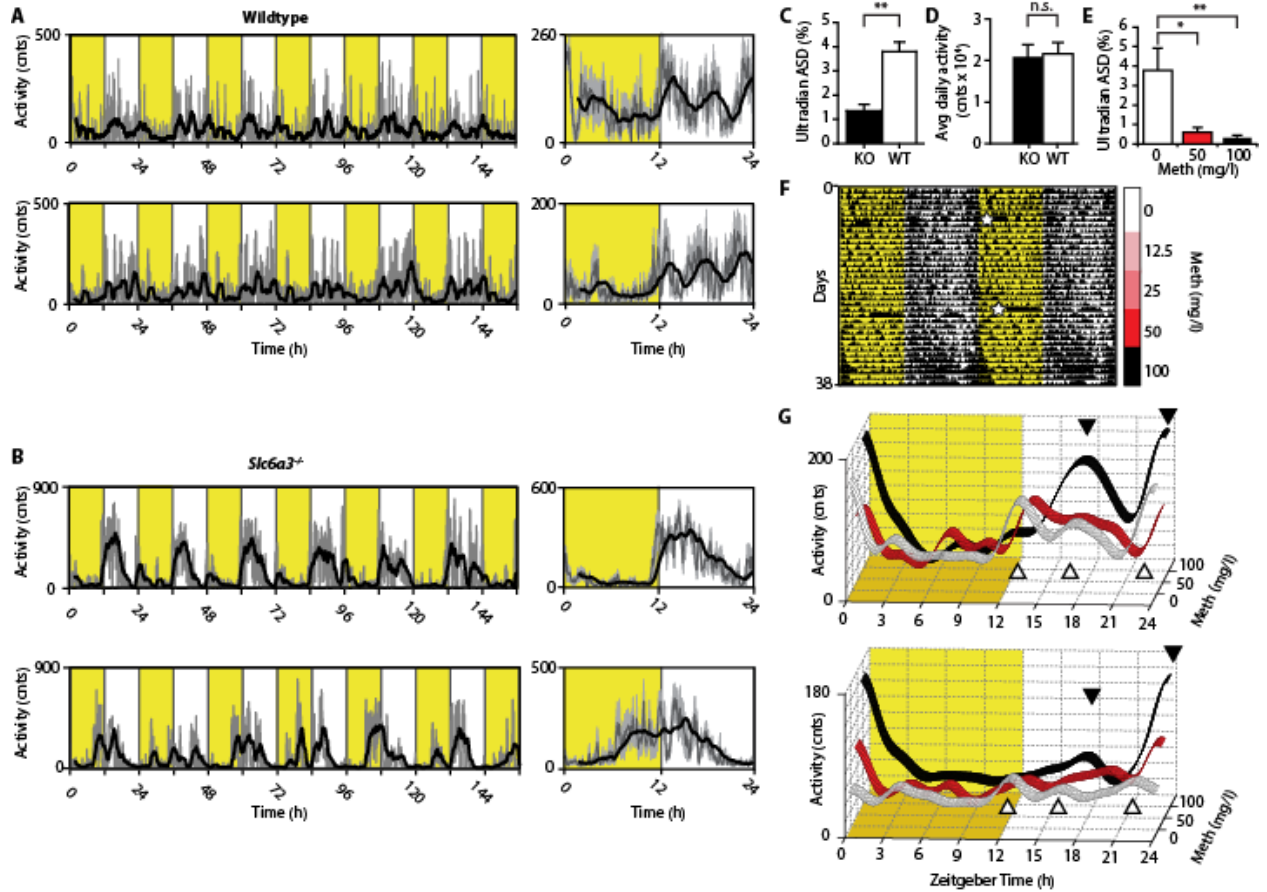


Figure 12: Ultradian activity in SCN-intact *Slc6a3*^{-/-} mice and their wildtype littermates. **(A and B)** Ambulatory activity recorded by telemetry implants across multiple days (left) and averaged over 24 hrs (right). Traces represent 2-h recursive smoothing (black) of the underlying raw DATA (dark grey; SEM envelope, light grey). Areas in yellow indicate lights on. **(C)** Amplitude spectral density in the ultradian range (2 to 8 hrs) is significantly different between *Slc6a3*^{-/-} mice and their wildtype littermates revealing a loss of the ultradian component (mean $\pm$ SEM; N=10; $F_{1,18}=26.40$, $**P<0.0001$, ANOVA). **(D)** Although the temporal pattern of locomotion differs between genotypes there is no significant difference in daily activity averaged over multiple days (mean $\pm$ SEM; N=10, $F_{1,18}=0.1793$, ANOVA) **(E to G)**, Addition of Meth to the drinking water of C57BL/6 mice lengthens the night-time activity bouts in a concentration-dependent manner. Averaged daily locomotor activity of individual mice at different Meth concentrations (G) derived from the time-span indicated by colored bars next to the representative actogram (F) and subjected to Butterworth filtering. The three night-time activity peaks before treatment (white triangles), transform to 2 peaks after exposure to the highest concentration (black triangles). The night-time bout lengthening is also reflected in the reduction of ultradian amplitude spectral density in the 1 to 5-h range (E, mean $\pm$ SEM, N=7; $F_{2,20}=8.08$, $P<0.005$, repeated measures ANOVA; $*P<0.05$, $**P<0.01$, post-hoc Bonferroni). White asterisks (in E) indicate cage changes.

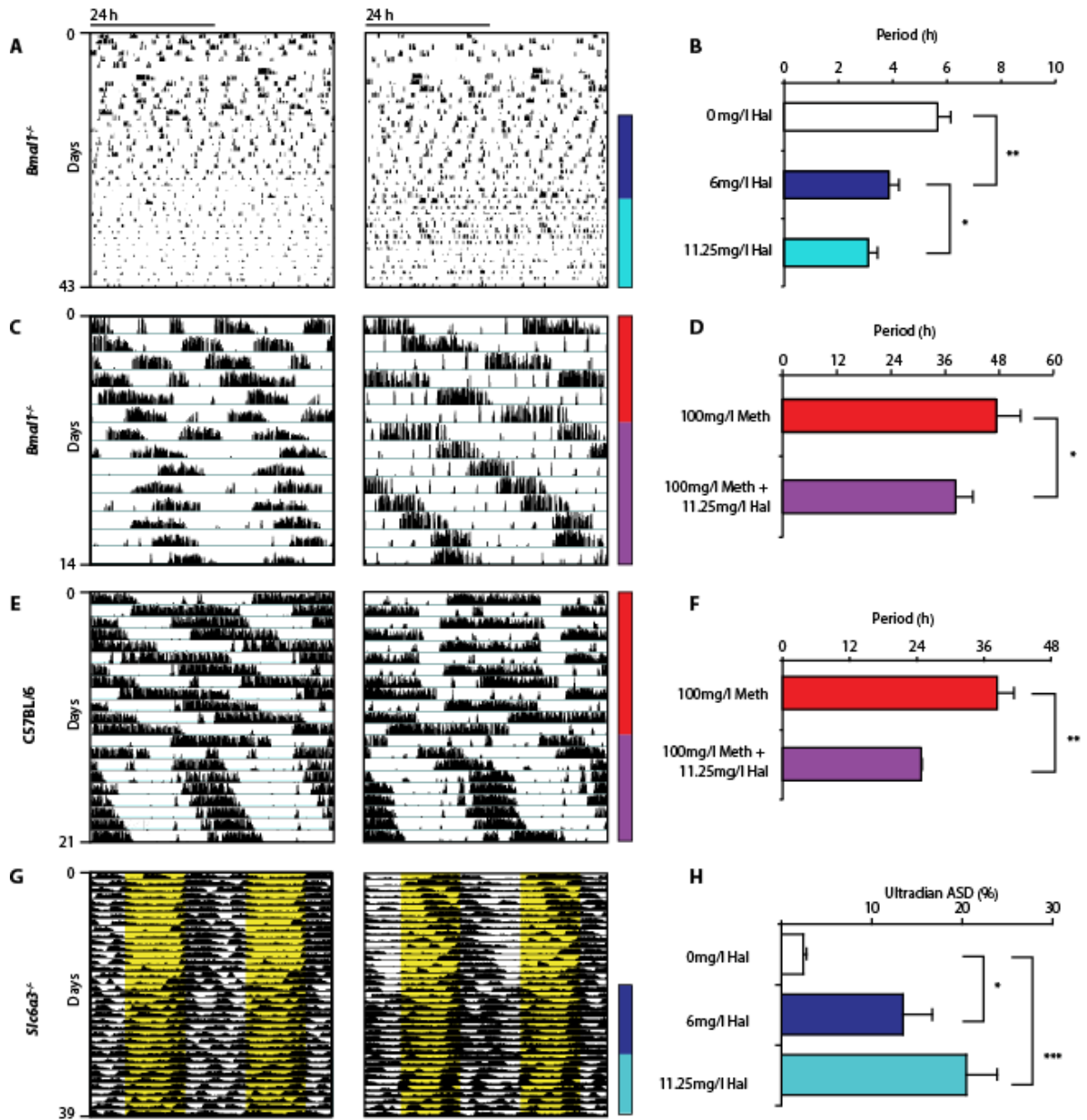


Figure 13: Haloperidol shortens circadian-clock-independent locomotor rhythms. Representative actograms with Hal treatment periods indicated by colored bars (*left*); bar graphs indicate corresponding locomotor period (*right*). (**A**) Increasing concentrations of Hal provided in the drinking water gradually shortens the endogenous ultradian activity rhythms of *Bmal1*^{-/-} mice in DD (**B**, mean $\pm$ SEM, N=12; $F_{2,10}=14.36$, $P<0.0001$, repeated measures ANOVA; $*P<0.05$ and $**P<0.01$, planned comparison ANOVA). (**C** and **E**) Hal shortens the infradian rhythms in Meth-treated *Bmal1*^{-/-} mice (**D**, mean $\pm$ SEM, N=9; $F_{1,8}=2.357$, $*P<0.05$ ANOVA) and WT mice (**F**, mean $\pm$ SEM, N=9; $F_{1,8}=3.525$, $**P<0.01$, ANOVA) in DD. (**G**) Hal treatment increases the frequency of temperature fluctuation in *Slc6a3*^{-/-} mice under LD measured by telemetric implants. (**H**) Changes of amplitude spectral density in the ultradian range (2-8 hrs) in response to increasing Hal concentrations (mean $\pm$ SEM; N=8; $F_{2,7}=14.74$, repeated measures ANOVA, $*P<0.05$, $***P<0.0005$, post-hoc Bonferroni). For all experiments, periods are calculated based on the running wheel activity during the last seven days of treatment at the indicated Meth/Hal concentration.

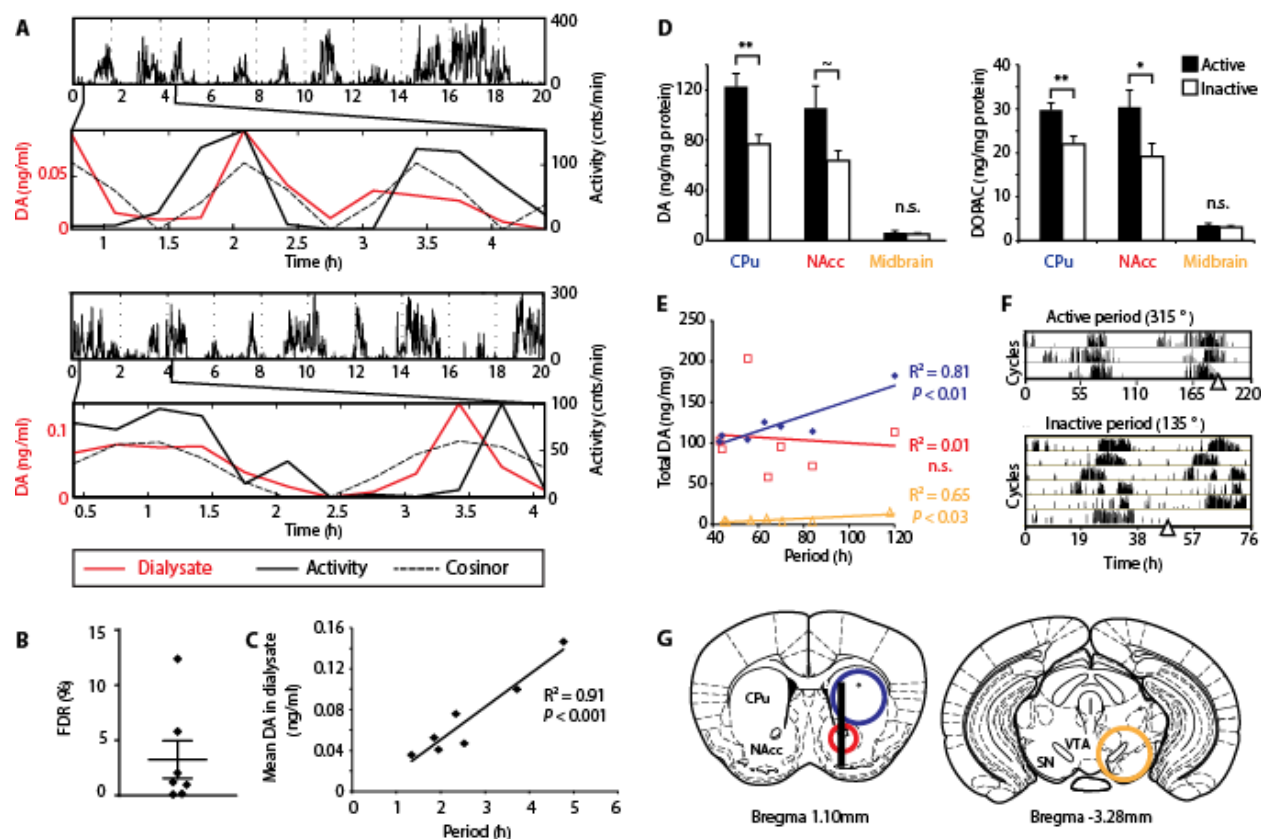


Figure 14: DA fluctuations correlate with ultradian locomotor behaviour in *Bmal1*^{-/-} mice. **(A)** Two representative examples of in vivo striatal microdialysis in *Bmal1*^{-/-} mice. Upper: locomotor activity as measured by beam breaks. Lower: DA dialysate concentration measured at 20-min intervals (red trace) plotted alongside the corresponding locomotor activity (solid black trace). Cosinor (dotted line) was computed from the 20 hrs of locomotor activity following dialysate sampling. **(B)** False discovery rate of the fit between the DA profiles and corresponding cosinors (mean $\pm$ SEM, N=7). **(C)** Linear regression analysis of period length versus mean DA concentration, dots representing individual animals. **(D)** Tissue punches of CPu, NAcc, and midbrain were analyzed for DA and DOPAC content in animals sacrificed during their active (■) versus their non-active (□) phase (mean $\pm$ SEM; active phase, N=7, inactive phase, N=6; * $P < 0.05$, ** $P < 0.01$, ~ $P = 0.066$, ANOVA). **(E)** Linear regression for period versus DA content in animals sacrificed during the active phase revealed significant correlations for the CPu and midbrain. **(F)** Representative actograms displaying activity rhythms of Meth-treated *Bmal1*^{-/-} mice used for tissue collection. Triangles indicate collection time points (in °), with locomotor activity bout onset set to 180°. **(G)** Illustrations of coronal mouse brain sections (294) indicating position of the active membrane (black bar) and tissue punch placements (circles, colors correspond to labels in D and E).

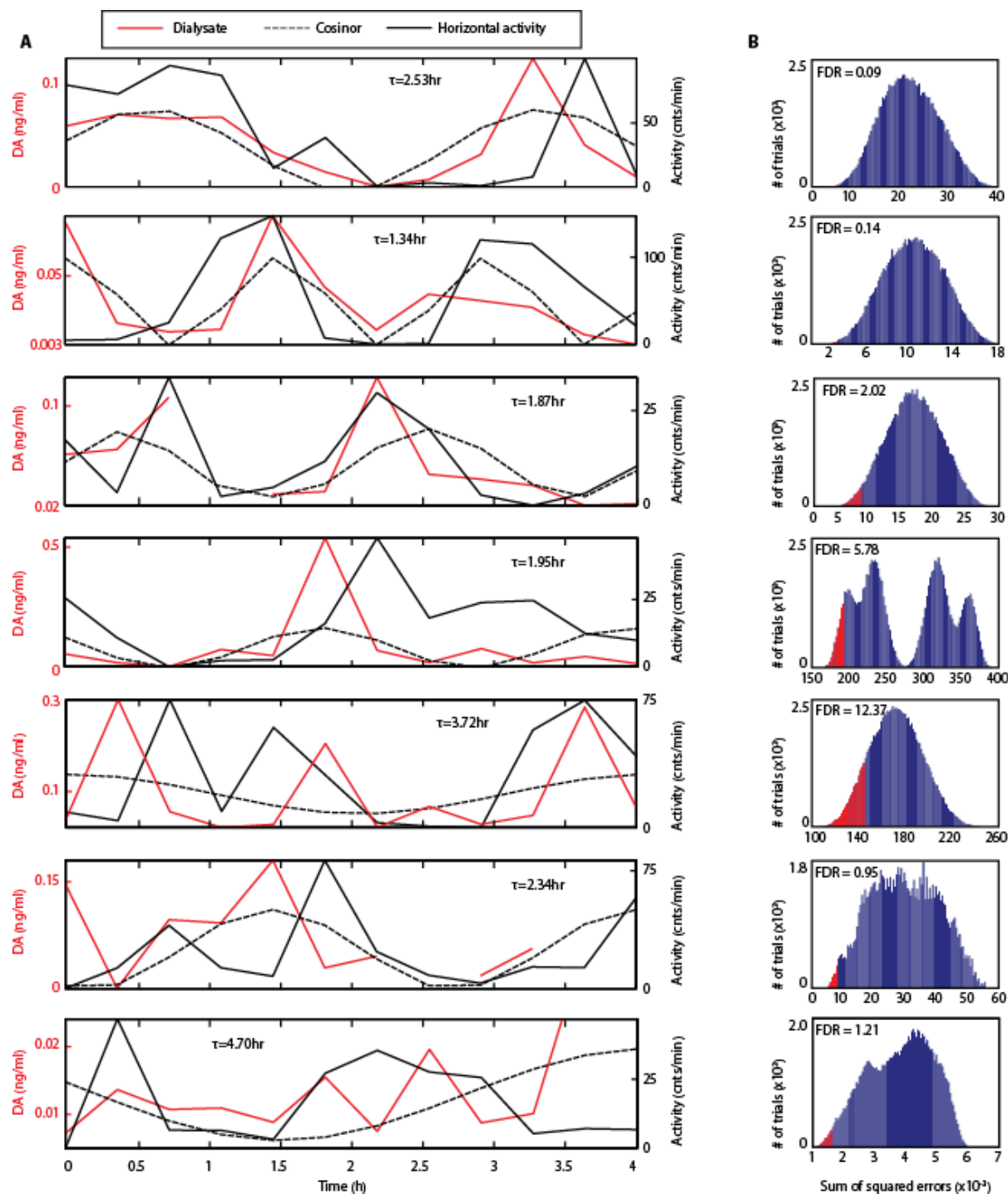


Figure 15: Rhythms of extracellular DA in the striatum of freely-moving *Bmal1*^{-/-} mice. **(A)** Individual records of striatal in vivo microdialysis showing DA dialysate concentrations (red), horizontal activity in 20-min bins (black) and the cosinor trace (dotted). τ indicates locomotor rhythm period computed from the 20 hrs of horizontal activity recorded after dialysate collection. **(B)** Histogram plots showing the distribution of the summed square of errors values for the fit between the cosinor and the DA profile after random permutation of the latter (100,000 operations). False discovery rate is calculated by determining the percentage of permuted profiles which fit the cosinor as well or better than the experimentally determined DA profile (highlighted in red).

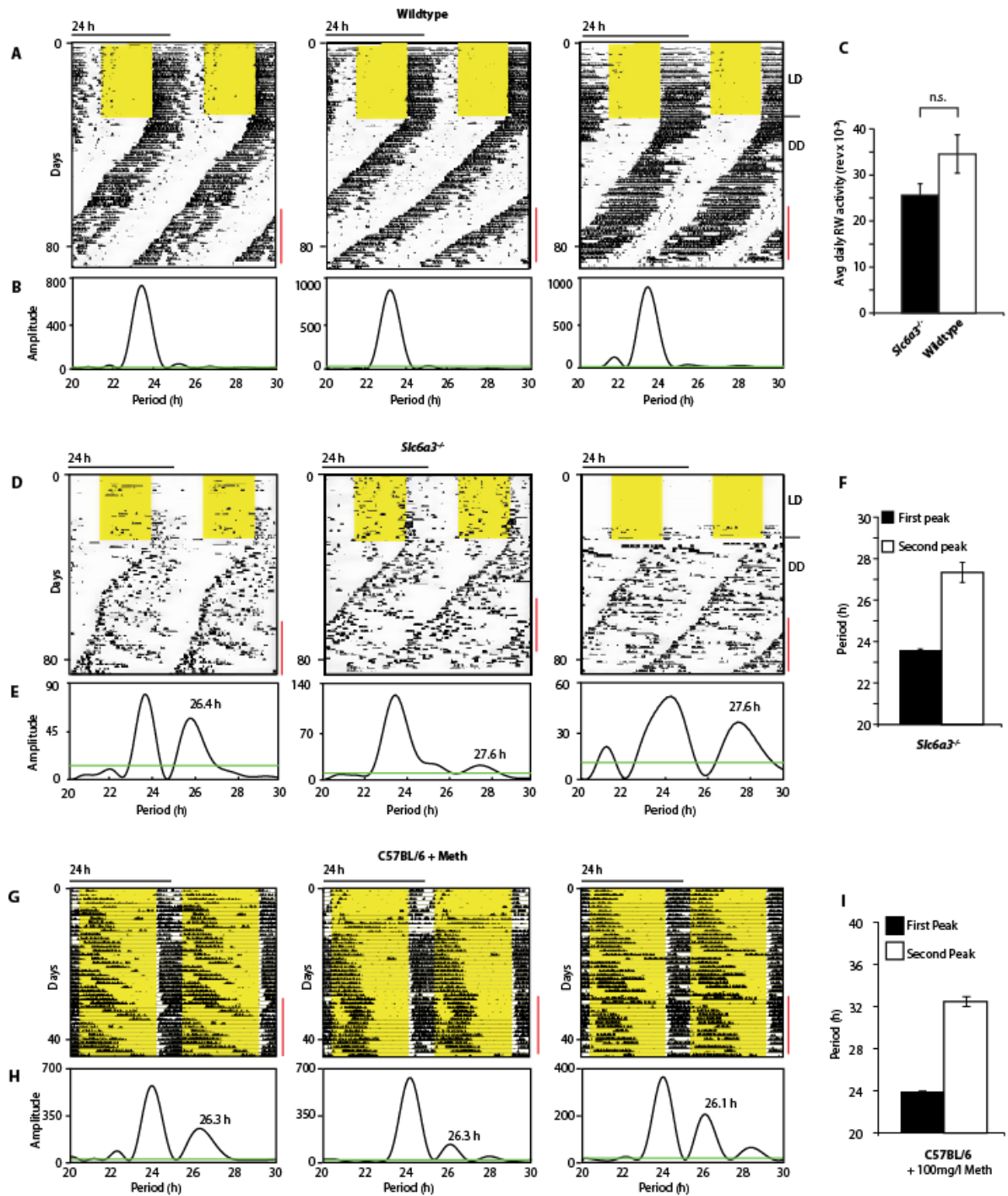


Figure 16: *Slc6a3*^{-/-} mice show a second rhythmic locomotor activity component. Representative actograms displaying daily running wheel activity of *Slc6a3*^{+/+} (A), *Slc6a3*^{-/-} mice (D) and C57BL/6 mice on Meth (G). (B, E and H) Lomb-Scargle periodograms generated from the time-span indicated by red bars, in (A), (C), and (G), respectively. (C) There is no significant difference between genotypes in daily activity averaged over the time-span of analysis (mean ± SEM; N=6, $F_{1,10}=1.848$, ANOVA) (F and I) Average periods of the highest 2 periodogram peaks for *Slc6a3*^{-/-} mice (E, mean ± SEM, N=6) and C57BL/6 mice on Meth (I, mean ± SEM, N=9, Meth-treatment started on day 1 of the recordings). Areas in yellow indicate lights-on. Green line in the periodograms indicates the confidence threshold for rhythmicity ($\alpha=0.01$).

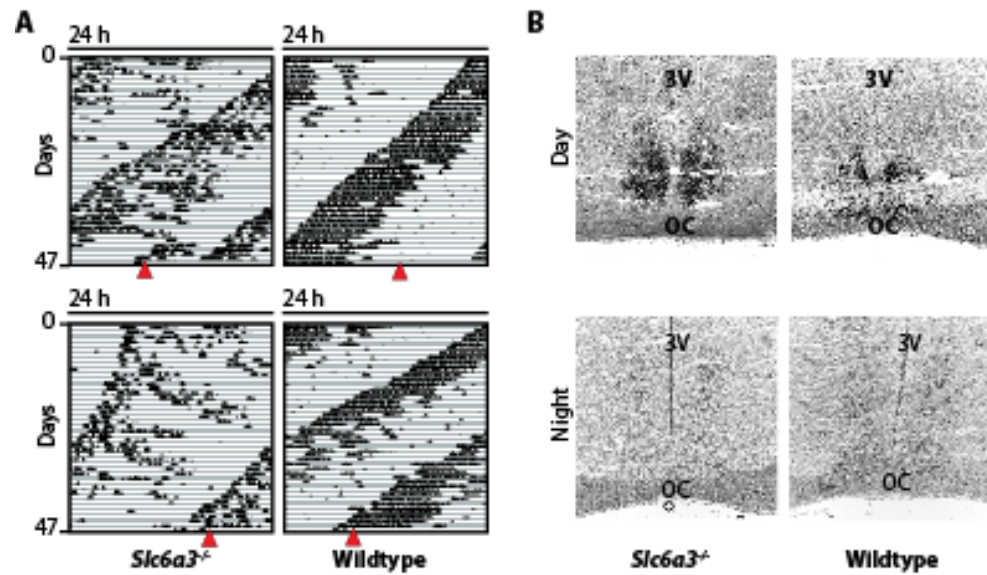


Figure 17: The SCN of *Slc6a3*^{-/-} mice is unperturbed (A) In situ hybridization indicates normal expression of *Per1* in the SCN of *Slc6a3*^{-/-} mice with strong staining of the SCN in the subjective day (Day) and barely detectable probe signal in the subjective night (Night), a pattern similar to wildtype SCN. (B) Subjective day and night were determined based on running wheel records using the onset of the circadian alpha band. Red triangles indicate time of sacrifice. 3V, third ventricle; OC, optic chiasm.

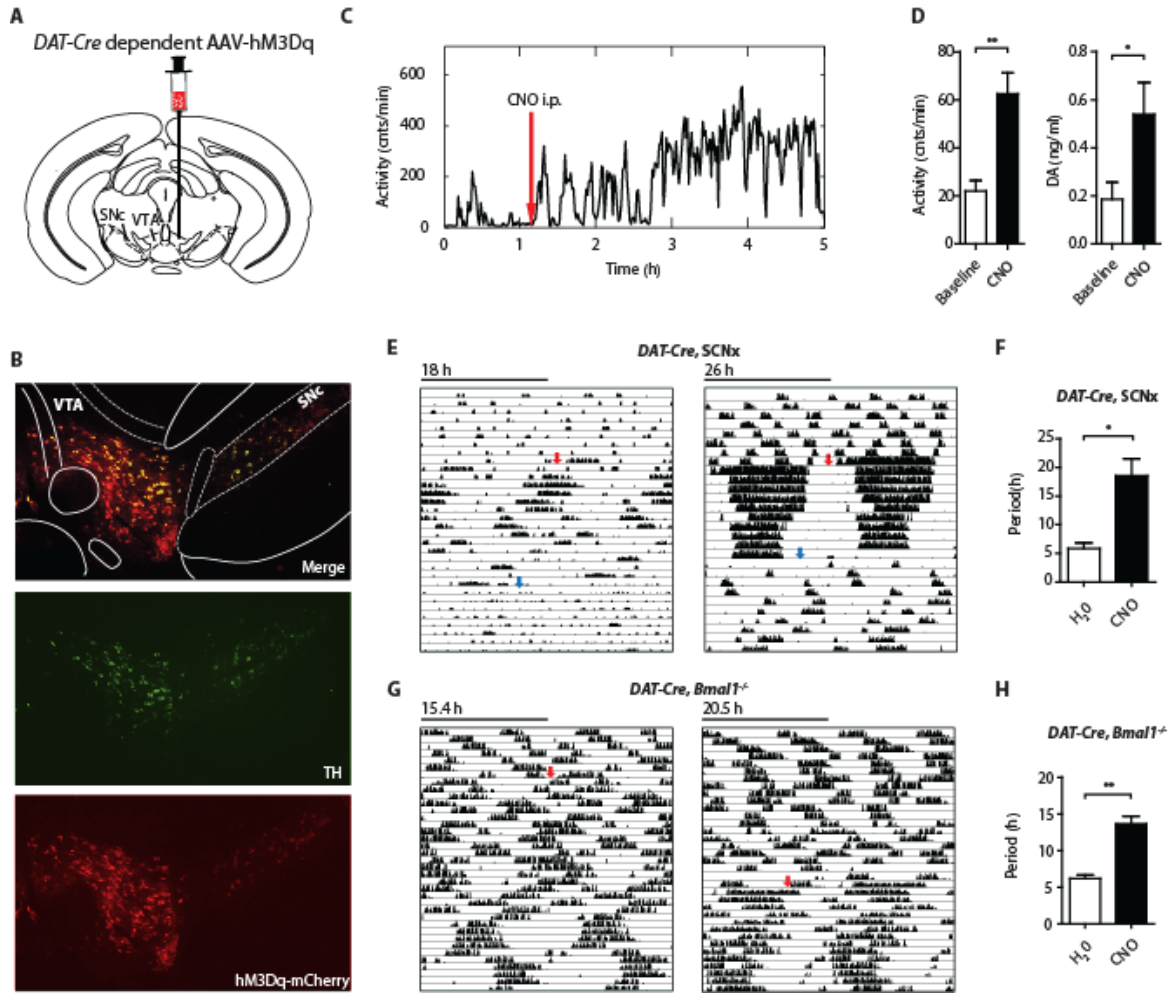


Figure 18: Chemogenetic activation of midbrain DA neurons lengthens ultradian locomotor period. (A) AAV-DIO-hM3Dq-mCherry was stereotactically and bilaterally delivered into the VTA/SN region of *DAT-Cre* transgenic mice as indicated. (B) Representative immuno-fluorescent image of the ventral midbrain from a virus-injected and behaviourally responsive mouse showing extensive co-expression of the mCherry fusion-tag in TH-positive cells of the midbrain. (C) Locomotor response to CNO (red arrow, 1mg/kg body weight i.p.) of a representative, AAV-hM3Dq transduced *DAT-Cre* mouse undergoing microdialysis. (D) 20min- binned locomotor activity and extracellular striatal DA content (20min) of AAV-hM3Dq transduced mice 1 hr prior (Baseline) and 2 hr after CNO injection. Mice were implanted with a striatal microdialysis probe and DA content was measured as in Figure 6A (mean $\pm$ SEM; N=3, * P <0.05, ** P <0.01, paired t-test). (E to H) Representative actograms of AAV-hM3Dq transduced *DAT-Cre*, SCNx (E) and *DAT-Cre*, *Bmal1*^{-/-} mice (G). Switch to CNO-containing drinking water (7.5mg/L) is indicated by red arrow. Blue arrow marks return to regular water (in E). Animal activity is plotted modulo according to the period measured during the last 7 days of CNO treatment. Periodogram analysis reveals CNO-dependent period lengthening in both *DAT-Cre*, SCNx (F, mean $\pm$ SEM; N=6, $F_{1,5}$ =3.68, * P <0.05, ANOVA) and *DAT-Cre*, *Bmal1*^{-/-} (H, mean $\pm$ SEM; N=11; $F_{1,10}$ =20.48, ** P <0.0001, ANOVA) mice. VTA, ventral tegmental area; SNc, substantia nigra pars compacta.

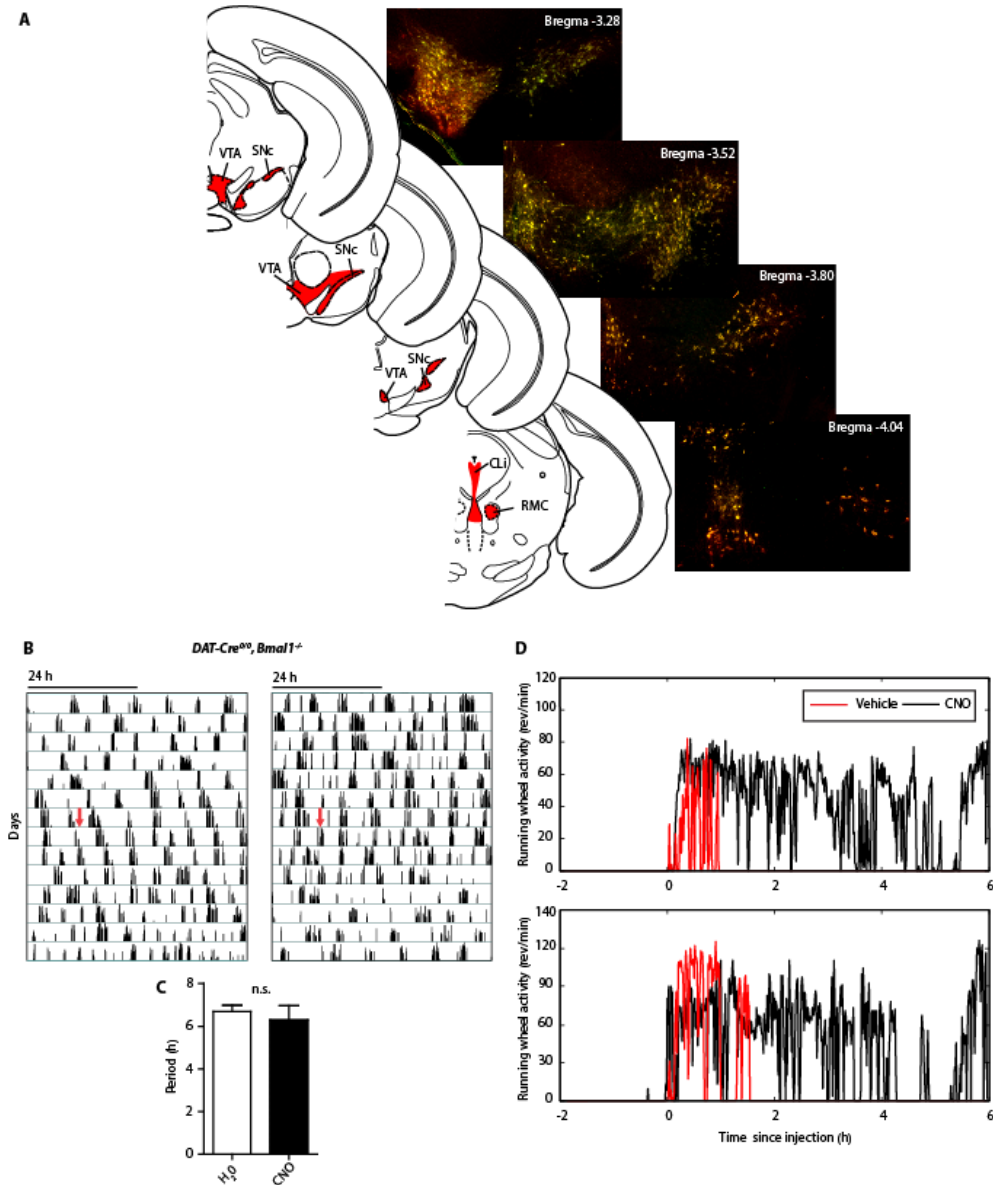


Figure 19: *AAV-h3MDq* targeting of DA neurons. **(A)** Midbrain expression pattern of mCherry and tyrosine hydroxylase (TH) in *DAT-Cre* mice upon bilateral injection with *AAV-DIO-h3MDq-mCherry*. Shown are immunofluorescent images of representative sections of CNO-responsive mice that were stained with dsRed (red) and TH (green) antibodies demonstrating comprehensive h3MDq-mCherry expression in TH neurons. Corresponding illustrations indicate sites of viral expression (red). **(B)** *AAV-h3MDq* injected *DAT-Cre^{0/0}, Bmal1^{-/-}* mice which lack the *DAT-Cre* transgene do not exhibit a change in ultradian activity when exposed to CNO. Shown are representative actograms of running wheel activity in DD. Red arrow indicates switch to CNO (7.5mg/l) containing drinking water. **(C)** Periodogram analysis indicates no significant difference in locomotor period between pre and post-treatment (mean $\pm$ SEM; N=7, $F_{1,6}=0.401$, ANOVA). **(D)** Acute locomotor response (running wheel activity) of *AAV-h3MDq* injected *DAT-Cre* mice to midday (ZT 6) i.p. injections of either vehicle (0.5% DMSO in 0.9% Saline) or CNO (1mg/kg b.w. in vehicle). VTA, ventral tegmental area; SNc, substantia nigra pars compacta; CLI, caudal linear nucleus of the raphe; RMC, red nucleus, magnocellular part.

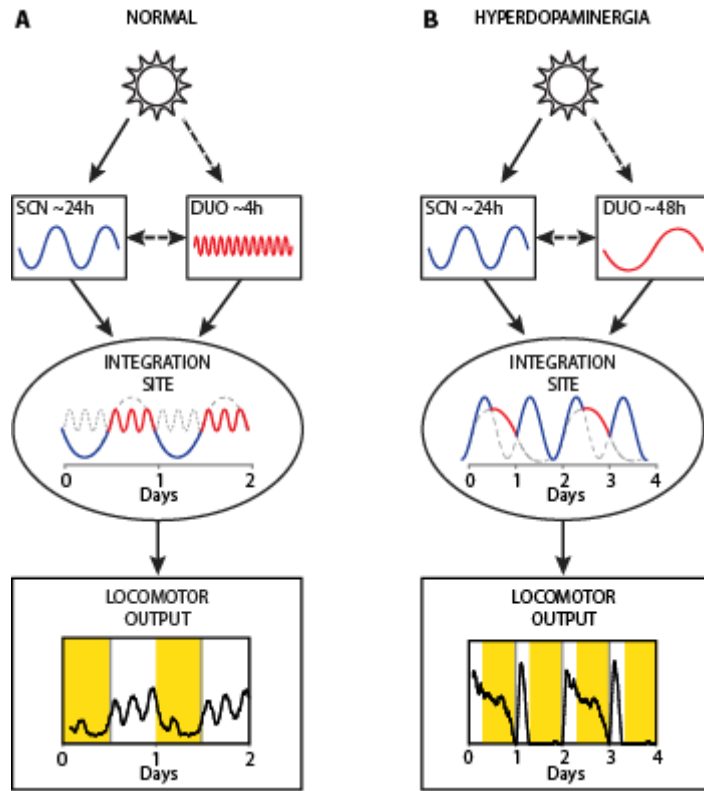


Figure 20: Proposed model of circadian and ultradian oscillator output integration to govern daily locomotor behaviour. **(A)** Light input entrains the circadian (SCN) and (indirectly) the ultradian (DUO) oscillators creating a stable phase relationship. Their rhythmic outputs, upon integration at a common downstream effector, generate the daily pattern of locomotor activity. **(B)** Under conditions of high DA tone (e.g. DAT elimination or Meth-treatment), DUO period lengthens, leading to a second, separate activity rhythm. This rhythm either free-runs (see Figure 7D and G) or phase locks with the SCN pacemaker by adopting a subharmonic, i.e. 48-h period, as frequently observed upon Meth treatment. Representative output plots show average activity of individual mice computed from eight days of ambulatory activity (A) or fourteen days of running wheel activity under Meth treatment (B).

Chapter 4: Evidence for ultradian behavioural rhythmicity reliant on intracellular feedback within dopamine neurons

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Abstract:

With the recent characterization and localization of a novel biological clock, the dopaminergic ultradian oscillator (DUO), several new questions emerge including the identity of the molecular or neuroanatomical substrate underlying rhythm generation. We hypothesized that the individual components necessary for self-sustained oscillations compose at least one feedback loop. By employing various genetic and pharmacological tools to disrupt these putative loops we hoped to reveal the underlying clockwork mechanisms – copying a strategy which has been extremely successful in the study of other biological oscillators including the circadian molecular clock. By systematically eliminating known extrasynaptic and extracellular feedback loops we demonstrate that not only are the other monoaminergic systems dispensable, but dopamine D1 and D2 are not required for rhythm generation. Finally, D2-autoreceptors, expressed on dopamine cells themselves, are not necessary. We then provide pharmacological evidence that DUO oscillations rely not on extracellular dopamine levels, as previously suggested based on correlations between dopamine dialysate and locomotor period, but rather it is intracellular feedback which may be critical. This suggests that self-sustained oscillations of dopamine are cell-autonomous and opens a new path for the investigation of this novel clock, one which leverages *in vitro* modelling to more quickly and efficiently screen for molecular targets important for DUO function and dysfunction alike.

Introduction:

A critical feature of all known biological oscillatory processes is some type of feedback loop. Interestingly, there are many ways that feedback can occur in natural systems and indeed for every seemingly possible scheme, there appears to be an example of a clock or timing system. A prime example is the circadian clock. It relies on transcriptional-translational feedback loops (TTFLs) in all eukaryotes e.g. fungi like *neurospora* (295), plants like *arabidopsis* (296), invertebrates like *drosophila* (297), and mammals like mice (23). However there are reports of many other kinds of biological oscillators that do not fit the standard TTFL model (84, 298), which are so highly studied because of advances in genetic tools allowing creative scrutiny. These include the proven cyanobacterial (299) and postulated dinoflagellate (300) phosphoprotein-oscillators, reduction-oxidation clocks in alga (86) and mammalian red blood cells (87). There are even many purely chemical oscillators like the Belousov-Zhabotinsky (301) or Briggs-Rauscher (302) iodine clock reactions, all of which critically rely on negative feedback. In each case, there is a network of interacting molecules, of varying size and complexity, performing roles including maintaining rhythm generation, setting the timing of the oscillations, entrainment to external stimuli, and providing precision and robustness to the network. Of particular importance to our own investigations presented here is the maintenance of rhythm generation, which typically constitutes two parts acting in direct opposition. The first – a positive-limb—is a mechanism which drives the clock inexorably forward and the second—a negative-limb—contains (a) feedback mechanism(s) acting on the positive driver to halt the clock and beginning each cycle anew. Without each of these two components cycling will not occur. In some oscillating networks, such as the circadian system, there are multiple feedback loops, which add not only precision and finer control, but also robustness against a loss of a single component (303). To complicate matters further, self-sustained rhythmicity, otherwise known as stable limit cycles, of the kind observed in biological oscillators also rely on some delay in the feedback-loop which sets or otherwise tunes the period of these oscillations(79). Without a delay component these cycles would quickly dampen (or expand) and reach a steady state (for a fascinating primer within a biological context see 304).

As we have gathered critical evidence for the existence of a novel biological oscillator within the dopaminergic system of mammals (158), one that is self-sustained and crucially relies on dopamine in the central nervous system (see Figure 21 for a recap), we can now attempt to investigate the mechanistic basis of these oscillations. Guided by lessons from other biological

oscillators, chiefly the circadian system, we can begin to probe the dopaminergic ultradian oscillator (DUO) by noting the distinct changes in oscillator properties (like period, phase, and amplitude) after specific biochemical perturbations. Therefore we hypothesize that the observation of perturbed locomotor rhythmicity in mice after various pharmacological and genetic manipulations can help to map out the distinct nodes of the dopaminergic oscillatory network.

Clearly the dopamine transporter (DAT) is not an essential node of the feedback loop as it's role is to change the period of the oscillator by gating the re-entry of dopamine back into the cell, instead accumulating it in the synaptic and extracellular space. It is the presence of dopaminergic oscillations in the absence of the DAT altogether (in knockout mice) which proves this point (158). In order to be a critical node, whatever proteins or molecules are involved must have direct, and opposing, action on the positive driver and hence their removal would not simply result in period changes (as might be expected for changes to a delay mechanism) but must halt the oscillations altogether. Similarly, our work with chemogenetic neural activation of dopamine cells, using DREADDs, suggests that synaptic release might also play a critical role in mediating the delay parameter, by sequestering free dopamine from either acting on a putative extracellular feedback node or preventing it from being reuptaken into the cytoplasm where it may similarly act to feedback on some key process. Intriguingly, haloperidol treatment (a dopamine receptor 2 inverse agonist) reduced the period of the dopaminergic ultradian oscillator but since the complete removal of the receptor was never tested, its necessity for feedback is currently unknown.

One might expect redundancy in such a critical network suggesting that finding a single critical node in the DUO is unlikely. However that same argument could be made for the circadian system and yet there is at least one player which topples the system when removed, *Bmal1* (21). Furthermore removal of individual feedback components, even in a highly redundant network such as the mammalian TTFL (50) has striking consequences for sustained rhythmicity, some of which might be compensated for by neural networks rather than molecular ones (305). Perhaps redundancy in this case is not important for either system (i.e. the circadian pacemaker and the DUO) as they can partially compensate for the loss of the other? This is at least true for the loss of the circadian timer (152, 231, 236, 306), but might it also be true for the DUO? Loss of dopamine transmission is clearly untenable (307), and hence unlikely to underlie observable cases of DUO dysfunction, at least outside of an experimental context. However, loss of DUO rhythmicity does not necessarily imply that dopamine production or release stops all together. If perturbations of the

network act on the negative limb, especially if they somehow remove feedback altogether, this may result in the oscillator being clamped at its peak rather than at its trough. This would allow dopamine production/release to continue relatively unabated, exclusively under circadian control (268, 269, 308, 309), but may have some serious behavioural or physiological consequences because of chronic (hyper?)dopaminergic tone. Some of these effects including mania, and even psychosis have been characterized in abusers of meth(amphetamines) or cocaine (310) and even modeled in DAT knockout (311) or knockdown (312) mice, however the underlying aetiology of hyperdopaminergia may vary widely at both the genetic and systems level. Therefore, we hypothesize that the negative-limb of the DUO is likely sole target of viable exogenous or endogenous perturbations leading to aberrant dopaminergic rhythmicity in vivo. Therefore feedback loops will be the primary target of our current investigations.

Results and Discussion:

Reciprocal connectivity between all midbrain monoaminergic nuclei

The dopaminergic system (DA, Ventral tegmental area) projects to both the serotonergic system (5-HT, Median and Dorsal Raphe) and the noradrenergic system (NorE, Locus Coeruleus) as visualized by tritiated leucine/proline tracing (313) and fluorescent retrograde tracers (314). Conversely dopaminergic cells receive serotonergic synaptic input visualized by electron microscopy of tritiated 5-HT (315-317) and these inputs arise from both the dorsal and median raphe as assessed by retrograde fluorogold tracing (318). Functionally the inhibitory role of 5-HT on dopamine release has been widely studied with a distinct relevance for psychopathology (for review see 319). 5-HT neurons also project to the noradrenergic LC (320) with tryptophan hydroxylase expressing nerve terminals within this nucleus (321, 322), exerting an inhibitory influence on the LC (323, 324). Finally, LC neurons are also known to project to dopaminergic neurons within the VTA (318, 325), and serotonergic neurons in both the dorsal and median raphe (326, 327). Intriguingly, the (somewhat oversimplified) network which emerges from the interrelations of these monoaminergic nuclei forms a model for neuroanatomical feedforward and feedback loops (NFFLs, Figure 22A), a type of structure strikingly similar to those found in most biological oscillators. In support of this, spectral analysis of cerebrospinal fluid serotonin metabolites reveals a significant increase in both 90-min and 4-h rhythms during the nighttime after antidepressant treatment suggesting that 5-HT plays a role in ultradian rhythmicity (328). Therefore, we hypothesized that the DUO might require an

extrasynaptic feedback loop composed of the major monoaminergic nuclei. We set about to test the contribution of both serotonin (5-HT) and Norepinephrin (NorE) to DUO rhythmicity by selectively ablating their vesicular packaging, and hence synaptic release, in the brain and then measuring ultradian locomotor periodicity as a correlate of dopamine oscillations and the output of the DUO (158).

Vesicular monoamine transporters package biogenic amines into intracellular storage organelles such as synaptic vesicles, shielding them from degradation and appropriately concentrating them for quantal synaptic release. They exist in two isoforms: VMAT1 is mainly expressed in chromaffin and enterochromaffin cells of the periphery, while VMAT2 is essentially expressed in monoaminergic neurons (329). Neonatal VMAT2^{-/-} mice do not release biogenic amines however they do not survive beyond the first postnatal days, preventing long-term evaluation of 5-HT or Norepinephrin release (330, 331). This limitation was overcome by Narboux-Neme et al. (332) through the generation of conditional (floxed) VMAT2 mice. These mice can then be crossed against Cre-recombinase expressing mice in which the driver is either the serotonin reuptake transporter (*Slc6a4*, aka SERT) or dopamine beta hydroxylase (*Dbb*, a key enzyme in the norepinephrine biosynthesis) to allow for the specific and restricted loss of either serotonin or norepinephrine vesicular packaging and hence release.

In order to best quantify any potential effects of monoaminergic manipulation upon DUO rhythms, we must first eliminate the overwhelming influence of the circadian system on locomotion. This is accomplished via electrolytic lesioning of the master circadian pacemaker in the brain, the suprachiasmatic nucleus of the hypothalamus (SCN). We have previously shown that not only does the removal of this structure allow for better visualization and quantification of DUO rhythms but its elimination is equivalent to the whole-body genetic excision of the circadian clockwork at the cellular level, at least in terms of DUO period and amplitude (158). Therefore in order to investigate the role of either 5-HT or NorE for DUO rhythm generation, we placed *Slc6a4*^{Cre/wt} *Vmat2*^{flx/flx} and *Dbb*-Cre^{Tg} *Vmat2*^{flx/flx} in running wheels after ablation of the SCN and recorded their locomotor activity rhythms for at least two weeks (Figure 23). Interestingly there was a slight but significant increase of the free-running DUO period in the mice lacking VMAT2 in 5-HT cells compare to their wildtype littermates (Figure 23A). It should be noted that this difference is still well within the range of expected DUO periodicity at least observed across dozens of mice of varying ages but of the same congenic strain (C57BL/6J) and is certainly not an extreme phenotype like that of the

Slc6a3^{-/-} (dopamine transporter knockout) mice. Importantly the period lengthening observed here is consistent with the inhibitory role of serotonin on the release of VTA dopamine (333, 334), with increased period potentially explained by a significant increase in dopamine neuron spontaneous excitability and burst-length after selective loss brain serotonin release as has been observed in rats with serotonergic lesions (334). Therefore the period changes observed here may reflect a change in the state-variables of the DUO oscillator but not necessarily the abrogation of DUO oscillations, indicating that connectivity of serotonergic and dopaminergic cells does not form a feedback loop necessary for DUO oscillations. The observation of ultradian rhythms and furthermore a lack of period differences between *Dbh-Cre^{Tg} Vmat2^{flx/flx}* mice and wildtype littermates (Figure 23B) strongly suggest that LC NorE neurons do not form a positive feedforward loop, and therefore the NFFL as proposed (Figure 22A) is not responsible for self-sustained DUO rhythm generation.

Perhaps the existence of a NFFL, while meaningful for appropriate regulation of the circuitry involved, does not have the capacity to oscillate. As previously discussed, both a positive drive and negative feedback are necessary but limit cycle oscillatory behaviour also critically relies upon a delay mechanism for period determination and to prevent dampening to some steady-state. If a sufficient delay does not exist then this circuit may not contribute to intrinsic rhythm generation. Alternatively, if neuronal feedback within this circuit occurs much faster than the hours-long timescale of DUO oscillations, then it could help to rule out the putative NFFL as a mechanism for DUO rhythm generation and maintenance. Interestingly there is a dearth of evidence suggesting that this alternative is a more likely case with oscillations of raphé electrical activation occurring from seconds to minutes (for review see 335). Finally, we would be remiss if we did not mention a potential caveat here: it is possible, although never demonstrated to our knowledge, that vesicle-independent release (336) of 5-HT or norE contribute to extrasynaptic feedback loops of the DUO and by selectively manipulating vesicular packaging we did not appropriately test this, nor can we definitively rule it out as a putative mechanism for the DUO feedback loop until a better alternative is provided. The only argument we can make against it is the extremely powerful action of VMAT2 to sequester cytosolic amines (329), and the ~95% reduction of extracellular serotonin when VMAT2 is selectively knocked out (332); suggesting that any and all amine release must be the result of vesicular fusion, even if it can signal in a paracrine fashion.

The sheer breadth of dopamine modulating afferents (and hence putative extrasynaptic feedback loops) is staggering (for review see 337); too numerous to list here, let alone systematically investigate, for DUO dysfunction. However, any potential extrasynaptic feedback loops (Figure 22B) must rely on first order dopamine synaptic, or possibly even mean-field, transmission at dopamine effectors like the dopamine receptors. Two distinct families of genes coding for DA receptors exist. These are the D1-like (*Drd1* and *Drd5*) and the D2-like (*Drd2*, *Drd3*, *Drd4*) receptors which are responsible for all of the known downstream effects of dopamine release (338). In reality, the DRD3 (339, 340), DRD4 (341), and DRD5 (342, 343) have extremely low abundance in the brain and very restricted localization arguing for extreme specialization (344). Meanwhile, both the DRD1 and DRD2 have been implicated directly in the locomotor effects of stimulants like modafinil (345) and double transgenic mice lacking both receptors are neonatally lethal (346), with a significant decrease in the drive for locomotion or consummatory behaviour suggesting a critical role for these two receptors in particular for the DUO. Therefore, by using mice lacking these two major receptor subtypes, we were able to test the necessity of extrasynaptic feedback loops for DUO rhythm generation with only two simple experiments.

Electrolytic lesions of the SCN in *Drd1* and *Drd2* knockout mice and testing for the presence (and character) of ultradian oscillations of locomotion revealed no overt loss of ultradian rhythmicity in either line (Figure 24). Unfortunately, the phenotypic morphological variations of these particular mice generated significantly lower numbers of mice meeting our established inclusion criteria for SCN-lesion success. Due to this we could not statistically compare quantitative estimates of the period in the knockout mice with respect to wildtype counterparts. However, qualitatively these mice did not display any overt differences in locomotor rhythms (Figure 24), nor did the few successful lesions have oscillations outside of the period range (2-8 hrs) previously observed in SCN lesioned wildtype mice (158). This suggests that extrasynaptic feedback loops do not underlie DUO oscillations however we did not extensively account for redundancy. As these mice are genetically deficient at birth there is a possibility of compensation by one or another of the other D1-like or D2-like receptor, however this is theoretical only as there is no evidence to date from other behaviours or physiological markers that this might be the case, furthermore radiography using semi-specific ligands indicates that in each case of single dopamine receptor knockout mice there are no observed increases of closely related receptors (347). Furthermore, in terms of the D1-like

receptors, the DRD5 knockout mouse does show differences in locomotor responses in the open field and rotarod tests, however loss of the receptor increases locomotion (342) in agreement with a study using i.c.v. antisense oligonucleotides for the receptor (348). *Drd3*^{-/-} mice also show a hyperactive phenotype (340). These two cases are in direct opposition to the reduction of locomotion observed in *Drd1*^{-/-} or *Drd2*^{-/-} mice and therefore appropriate compensation by DRD5 in *Drd1*^{-/-} or DRD3 in *Drd2*^{-/-} mice is highly unlikely. The only remaining receptor which requires testing is the DRD4, as *Drd4*^{-/-} mice phenocopy *Drd1*^{-/-} and *Drd2*^{-/-} mice in terms of hypolocomotion (341). Paradoxically however these mice are supersensitive to cocaine, ethanol and amphetamines potentially suggesting abrogated inhibitory feedback mechanisms with respect to locomotion. That it is paradoxical perhaps suggests a role of the DRD4 as an autoreceptor however its expression is not observable in the rodent midbrain using antisera (349) or in situ hybridization (350) making this unlikely.

Dopamine autoreceptor feedback loops

Ruling out multisynaptic feedback as a putative mechanism for DUO rhythm generation we then turned to other potential sources of extracellular feedback. To date, the *bona fide* candidate identified for the feedback of dopamine onto dopaminergic cells is an autoreceptor of the D2-like family (**Figure 22B**), more specifically the dopamine receptor D2 (for review see 351). This receptor is expressed on presynaptic midbrain dopamine neurons of the VTA and SCN acting in three specific ways to presynaptically tune extracellular dopamine (reviewed in 352). First, DRD2s act via the G $\beta\gamma$ subunit to both activate inhibitory G-protein-activated inward rectifying potassium channels (GIRKs)(353) and inhibit voltage-gated calcium channels (VGCCs) (354, 355) at the membrane decreasing spontaneous neuronal firing. Second, these presynaptic receptors control cell surface localization of DAT as well as directly interacting as a heterodimer in order to increase its activity both of which modulate the rate of extracellular dopamine reimport after release (356, 357). Third, DRD2s act as G-Protein coupled receptors through G_{i/o} in order to inhibit adenylate cyclase (358, 359) which ultimately decreases PKA-mediated phosphorylation of tyrosine hydroxylase (TH) leading to decreased synthesis and vesicular packaging of dopamine (360, 361).

While it is unclear which, if any, of these mechanisms might mediate dopamine feedback on a timescale corresponding to DUO periodicity, we set out to test the involvement of these autoreceptors for rhythm generation in a manner very similar to those described above. In this

instance we used mice in which the DRD2 has been conditionally knocked out ($Drd2^{flx/flx}$) of dopamine transporter expressing neurons which transgenically express Cre-recombinase ($Slc6a3-Cre^{Tg}$). We first investigated these mice under a standard light-dark cycle and with an intact circadian systems in order to probe the necessity of D2-autoreceptors in the period shortening effects of haloperidol (a selective D2 reverse-agonist) which we previously observed in mice provided with ad libitum methamphetamines in their drinking water (158). Interestingly, both wild and conditional knockout mice showed obvious long free-running DUO rhythms after gradual increases in methamphetamine dose over several weeks and this could be visualized in either their body temperature oscillations (Figure 25A) or in weekly averaged waveforms of locomotor activity (Figure 25F). These observations were confirmed with an increase of the infradian ($>25h$) spectral density and a second periodogram peak well above 30-hrs on average (Figure 25B and C). In order to verify the loss of autoreceptors we performed an autoradiographic binding assay on brains from these same mice. Region of interest analysis demonstrates a distinct abrogation of D2 binding in the ventral midbrain VTA and SNc regions while striatal D2 is not significantly altered (Figure 25D and E). Of particular note are the three peaks of night-time activity in both genotypes under baseline conditions which we argue provide the first piece of evidence demonstrating intact DUO function in mice lacking D2-autoreceptors. Upon the addition of haloperidol to the drinking water (while still providing methamphetamines) there was a significant drop in the spectral density and the infradian period but this was crucially only observed in the wildtype mice carrying the D2-autoreceptor (Figure 25B and C). Therefore we conclude that the period shortening effects of haloperidol observed on the DUO act solely via the D2-autoreceptor. Finally, using a second cohort of drug naïve mice we lesioned the SCN to remove circadian interference and placed these animals in constant darkness to remove any exogenous timing cues. Upon release into running wheels we observed qualitatively similar locomotor oscillations in both genotypes (Figure 25G) strongly supporting our claim that the DUO is operative in mice lacking D2-autoreceptors and suggesting that extracellular feedback of dopamine is not critical for DUO rhythmicity.

Given the period altering effects of haloperidol, it is distinctly possible that the role of D2-autoreceptors is modulatory and not one of feedback *per se*. Of the three possible mechanism by which haloperidol may be acting through the DRD2, effects on electrical membrane properties are the least likely. It's effectiveness even when in the presence of large doses of methamphetamines, reversing the DAT transporter and downplaying the relative contribution of synaptic release,

strongly suggests that it either acts via second messenger signalling to reduce TH activity or by increasing DAT surface localization and/or activity. Potential methods for testing this in the future might be to measure extracellular dopamine levels or phosphoTH protein levels across time in these mice to look for changes after treatment with chronic haloperidol. Perhaps even the exogenous application of fluorescent false neurotransmitters and the measurement of subsequent reuptake could also resolve this question (362, 363) with respect to changes in DAT activity by haloperidol.

The first characterization of these mice demonstrated that tyrosine hydroxylase was almost twice as active in conditional knockouts and that this increased activity was insensitive to a D2 antagonist quinpirole. Furthermore, these mice were supersensitive to cocaine and synaptic release of dopamine was almost tripled after electrical stimulation (364). Taken together it is very surprising that we do not see endogenous period differences in our conditional knockout mice given that extracellular dopamine concentrations correlate so well with DUO periodicity. Perhaps our initial hypotheses were incorrect, and that the correlation was just that, correlative. The evidence accumulated thus far is mounting to suggest that perhaps it is not the extracellular concentrations of dopamine which are necessary for rhythm generation but rather that there is some intracellular process which might be critical, with intracellular levels normally correlating tightly with extracellular levels reliant on dopamine biosynthesis and the action of the dopamine transporter.

Evidence for intracellular feedback

Therefore we designed a set of experiments which would alter the correlation between extra- and intracellular dopamine levels in order to determine the true source of the DUO feedback loop. Pharmacologically speaking, there are at least two molecules which can act to reduce dopaminergic tone in different ways and both have been used alone or in combination in order to acutely reduce dopamine release (365). The first, α -methyl-*p*-tyrosine (α MPT), is a competitive inhibitor of tyrosine hydroxylase the rate limiting enzyme for dopamine and norepinephrine biosynthesis (366). The second, reserpine, is an inhibitor of vesicular monoamine transporters (367) reducing vesicular packaging and hence monoamine release. Importantly, while the neuroleptic effects of both of these drugs are attributable to reduce extracellular dopamine (Figure 26A), only α MPT reducing dopamine biosynthesis. Therefore the treatment of mice with these compounds in vivo should have disparate effects on the balance between extra- and intracellular dopamine (Figure 26B). We hypothesized that if extracellular feedback loops were critical for setting DUO periodicity,

then both drugs would have the same effect on rhythms of locomotion: period lengthening. Alternatively, the observation of divergent period changes after the application of these drugs strongly suggests that it is intracellular feedback which sets DUO period.

As a complete loss of dopamine release is the typical use of these drugs, alone or in combination, producing marked akinesia as a hallmark of effectiveness and our subsequent ability to observe DUO oscillations relies on the observation of locomotion, a sub-threshold dose for each was required. Therefore we set about to give once-weekly injections of either drug to mice lacking the critical circadian component BMAL1 exponentially increasing the dosage until intra-animal period effects were observable in locomotor traces (Figure 27A, D and G). We then quantitatively confirmed these transient period changes using Lomb-Scargle periodogram analysis, taking the highest peak in the ultradian range as our period estimate (Figure 27B, E and H). As a week should, theoretically, be enough time for either reserpine or α MPT effects to normalize we did not expect any order effects, nor problems with drug accumulation altering the repeatability of our final dosages. However in the name of caution, we repeated these same doses in these animals one week later and not only did the period of most mice start back at or near baseline levels, the direction and magnitude of period changes after drug were comparable (Figure 27C, F and I). Furthermore, the determined doses were also effective in naïve mice (far right, single trials). Together, the observation of period divergence upon drug treatment, with decreases in reserpine- and increases in α MPT-treated mice, strongly suggest that there is some intracellular feedback loop which tunes DUO rhythms. We are acutely aware of the potential for off target effects on other monoamine neurons using both of these drugs however given the lack of period changes with noradrenergic VMAT2 ablation and the opposing locomotor period effect of reserpine to that of VMAT2 deletion in serotonergic neurons, we are confident that the observed period effects are specific to their action directly on dopaminergic neurons themselves.

Finally, these results strongly support a novel hypothesis, that the period changes observed in all previous DUO manipulations was not due extracellular dopamine levels, but rather to changes of intracellular dopamine due to increased release (DREADDs), decreased production (α MPT) or decreased reuptake (DATKO, methamphetamines) for period lengthening and increased production (haloperidol) or decreased vesicular packaging (reserpine) for period shortening.

Conclusions:

Based on the manipulations of the monoaminergic systems presented here, either by 1) eliminating vesicular packaging of serotonin/norepinephrine, 2) genetically ablating downstream dopamine signalling (*Drd1*^{-/-}, *Drd2*^{-/-}), or 3) blocking extracellular feedback onto dopaminergic cells via D2-autoreceptors we suggest that extracellular dopamine is not a critical determinant of DUO feedback and hence may not be responsible for rhythm generation and maintenance of dopamine oscillations. This conclusion is strongly supported by our observation of divergent period changes after pharmacological dissociation of extra- and intracellular dopamine levels.

Interestingly, the application of a similar (but opposite) logic presented in the last set of experiments might resolve the mechanism of action responsible for period shortening effects of haloperidol. As previously discussed, haloperidol acts as an inverse-agonist on DRD2 receptors and its effects on period shortening are likely attributable to either the inhibition/reversal of PKA mediated effects on TH levels/activity or by directly inhibiting/altering the heterodimerization of DRD2-DAT to lower DAT surface localization and activity. In both cases, an increase of extracellular dopamine is likely; however the removal of membrane DAT, or the lowering of its activity, would result in a slowing of the buildup of intracellular concentrations of dopamine which should in turn lengthen the period of the DUO. From this we therefore suggest that it is the PKA mediated effects on TH which are the most likely ultimately contribute to the period shortening effects of haloperidol.

While our initial hypothesis was that extracellular dopamine levels determined the period of DUO oscillations due to their tight correlation, our investigations here systematically rule out that possibility and suggest another: that DUO oscillations rely on a putative intracellular feedback mechanism for rhythm generation and maintenance as this is the most parsimonious interpretation of all manipulations of the DUO to date. This novel hypothesis opens up a very interesting avenue for future exploration of DUO mechanisms: the possibility that these oscillations are cell autonomous and therefore may be modeled in vitro using either primary dopamine cell cultures or immortalized dopaminergic cell lines. In support of the conclusions drawn from our experiments presented here, our lab has already begun just such an undertaking, using the MN9D cell line (368), in conjunction with dopamine quantification based upon HPLC (369) and immunolabelling by an antibody that recognizes glutaraldehyde-conjugated dopamine (370). We do indeed observe oscillations of dopamine in these cells upon pre-treatment of reserpine to avoid washout of

dopamine upon extracellular release (personal communication with Dr. Lei Zhu). Importantly, this future line of investigations will allow for highly specific manipulations of intracellular processes and finer tools for the dissection of DUO rhythmicity in order to characterize the major (and minor) molecular substrate of these oscillations as it has for the circadian clock. It also argues for the need of tools which will allow us to follow DUO oscillations over time. Unfortunately dopamine, as a small molecule, is not amenable to tagging with fluorescent fusion proteins, or indeed proteins of any kind. Perhaps some hope resides in its biosynthetic pathway. If any of the key enzymes form a part of the core DUO clock, oscillating at the transcriptional level, then the use of promoter driven bioluminescent reporters might be possible as it is for the circadian clock. Therefore determining any genetic factors which contribute to DUO oscillations is of even greater importance, as some may hold keys to observing oscillations of this process at the single cell level.

The dynamic range of a particular monoaminergic transmitter's action on DUO rhythmicity, and the underlying mechanisms of said actions may be critical to defining the role of monoaminergic dysfunction in the aetiology and maintenance of dynamic, and possibly rhythmic, psychiatric diseases. Here we present significant advances in our understanding of the mechanisms critical for DUO regulation and rhythmicity with particular emphasis on the contributions of haloperidol to period shortening, the role (or lack thereof) of other monoaminergic systems for DUO dysfunction, and lay the groundwork for the DUO as a self-sustained, cell-autonomous oscillator important for the regulation of dopamine signalling.

Understanding the oscillatory network of the DUO and how each cog might influence its output may provide predictable behavioural correlates. Allowing clinicians and researchers to differentially diagnose psychiatric diseases from a behavioural and/or psychological phenotype (especially an oscillatory one) and given this information they will be able to more quickly and reliably trace these back to the target molecules and circuitry responsible.

Materials and Methods:

Animals *Bmal1*^{-/-} (21), *D1R*^{-/-} (371), *D2R*^{-/-} (372), *DAT*-iCre^{Tg} (261), *DRD2*^{flx/flx} (364), *SERT*^{Cre/wt} (373), *DBH*-Cre^{Tg} (KH212Gsat, 197), and *VMAT2*^{flx/flx} (332) mice were on a C57BL/6J genetic background. The Cre-loxP crosses used here have all been previously characterized with excellent recombination in their respective cell types; *SERT*-Cre X *flx*-*VMAT2* (332), *DAT*-Cre X *flx*-

DRD2 (372), and BHD-Cre X flox-VMAT2 (personal communication with Dr. Elsa Isingrini, manuscript in prep). Animals were housed under a 12h:12h light:dark cycle unless otherwise stated. Experimental procedures were performed in accordance with the Canadian Council on Animal Care guidelines and approved by the McGill University Animal Care Committee.

Amplitude spectral density (ASD) In order to determine the prevalence of infradian DUO oscillations (25-48h) we calculated the area under the curve of all significantly rhythmic periodicities ($\alpha = 0.01$) in the Lomb-Scargle periodogram, i.e. the spectral density. For normalization, the obtained spectral density was divided by the total significant spectral density in the 0 to 48-h range and expressed as percentage.

Autoradiography of D2 receptor binding Verification of autoreceptor deletion in DAT-iCre DRD2^{flx/flx} was carried out by using a [¹²⁵I] Iodosulpiride binding assay adapted from Martres et al. (372, 374). Briefly, mice were decapitated, and brains were rapidly removed and frozen in isopentane. Serial coronal sections (10 μ m) were cut at -20°C using microtome cryostat, thaw-mounted on Superfrost Plus slides and stored at -80°C until required. Slide-mounted brain sections were pre-incubated three times for 5 min at room temperature in 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ (tris-ion buffer) and supplemented with 10 μ M of Hydroxyquinoline and 0.1% ascorbic acid. They were then incubated for 60 min at room temperature in the same pre-incubation buffer with 0.2 nM [¹²⁵I]-iodosulpiride, with or without 1 μ M Raclopride (Sigma) to determine non-specific binding. Sections were then washed four times for 5 min each, in ice-cold Tris-ions buffer, rapidly rinsed in ice-cold water, dried and exposed to film (Biomax MR) for 20 h.

Butterworth filtering Low pass filtering (1-h cut-off) of raw data was conducted using a Butterworth zero-phase filter (291) in Matlab. This allowed for better visualization of ultradian frequencies in the >1 -h range, in a manner similar to recursive smoothing but without phase distortion.

Locomotor activity *Running wheels:* Animals were individually housed in light-controlled cabinets and activity was recorded continuously (ClockLab, Actimetrics). Actograms, displaying binned running wheel revolutions per 6 minutes (0.1 hr), and the associated Lomb-Scargle periodograms, displaying amplitude, were generated using ClockLab software. *Telemetry:* Animals were individually housed in standard cages placed atop energizer/receiver units (ER-4000, Mini Mitter). One week

prior to data collection, electromagnetic induction powered telemetry probes (G2 E-mitter, Mini Mitter) were implanted intraperitoneally. Locomotion, measured in counts per minute, and core body temperature (BT, in °C), was collected in 6-min bins (0.1 hr) using Vitalview software (Mini Mitter). BT data was exported into Clocklab to generate actogram-style data displays with tick mark height corresponding to temperatures from 34–38°C. Matlab (Mathworks) was used for ribbon plot generation, and low pass Butterworth filtering (1 hr).

Period determination Ultradian rhythms show variability in amplitude and period. We thus used Lomb-Scargle periodogram analysis (Clocklab) to estimate ultradian period length as this method is relatively tolerant to noisy data and data gaps, which can result from intermittent ultradian rhythm expression (292). Unless otherwise stated, period length was determined by identifying the highest peak above the significance threshold ($\alpha=0.01$) in the Lomb-Scargle periodogram. Subsequent plotting of an actogram at the determined modulus was conducted in each case in order to visually confirm rhythmicity and rule out the false identification of harmonic frequencies or side-lobes due to leakage (293).

Pharmacology α -Methyl-*p*-Tyrosine (α MPT, 100mg/kg, Sigma) for i.p. injections was prepared in 1M HCl with sterile saline (0.9%) then neutralizing the solution using a molar equivalent of 10M NaOH immediately prior to injection (375). Reserpine (0.3 mg/kg, Sigma) for i.p. injections was prepared by first dissolving in a single drop of glacial acetic acid (50 μ l) then diluting to the final concentration with sterile saline (376). 6-methyltetrahydropterin (6-MPH₄, 27mg/kg/day, Sigma) was prepared by dissolving in 0.1MHCl and neutralizing with 10M NaOH immediately prior to injection(377). A Stock solution of (+)-Methamphetamine hydrochloride (100mg/l, Sigma) to be provided in the drinking water was prepared using sterile filtered tap water (0.22 μ m), adjusted to pH 7 using sodium hydroxide, and stored at 4°C.

SCN lesions Electrolytic lesions of the suprachiasmatic nucleus were performed as described (199). Briefly, a custom designed electrode (Model UE-IB1, FHC) was lowered through a hole drilled in the skull at the mid-sagittal sinus according to stereotaxic coordinates (AP -0.40 mm, DV -6.00 mm from Bregma) and a constant current (2mA, 10s; D.C. Constant Lesion Maker, Grass Instruments) was applied. Only mice with behavioural circadian arrhythmia (in the 20 to 28-hr range assessed by Lomb-Scargle periodogram analysis) and subsequent post-mortem histological verification using DAPI staining mounting medium (Vectashield, Vector Labs) were included for analysis. This

represented approximately 50% of lesioned mice when averaged across all studies but was significantly less for the dopamine receptor knockout mice, perhaps due to morphological variation in these animals as the knockouts are sometimes overtly smaller than WT littermates. Furthermore, there was a marked increase in intracranial haemorrhaging in the SERT^{Cre}VMAT2^{flx/flx} mice. In subsequent cohorts the application of direct pressure using ophthalmic sponges to stem blood flow and then cauterization with a silver nitrate caustic pencil was effective in reducing attrition.

Statistical analysis ANOVA, t-tests, and linear regression analyses were performed using Prism 5 (GraphPad).

Acknowledgments

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Figures:

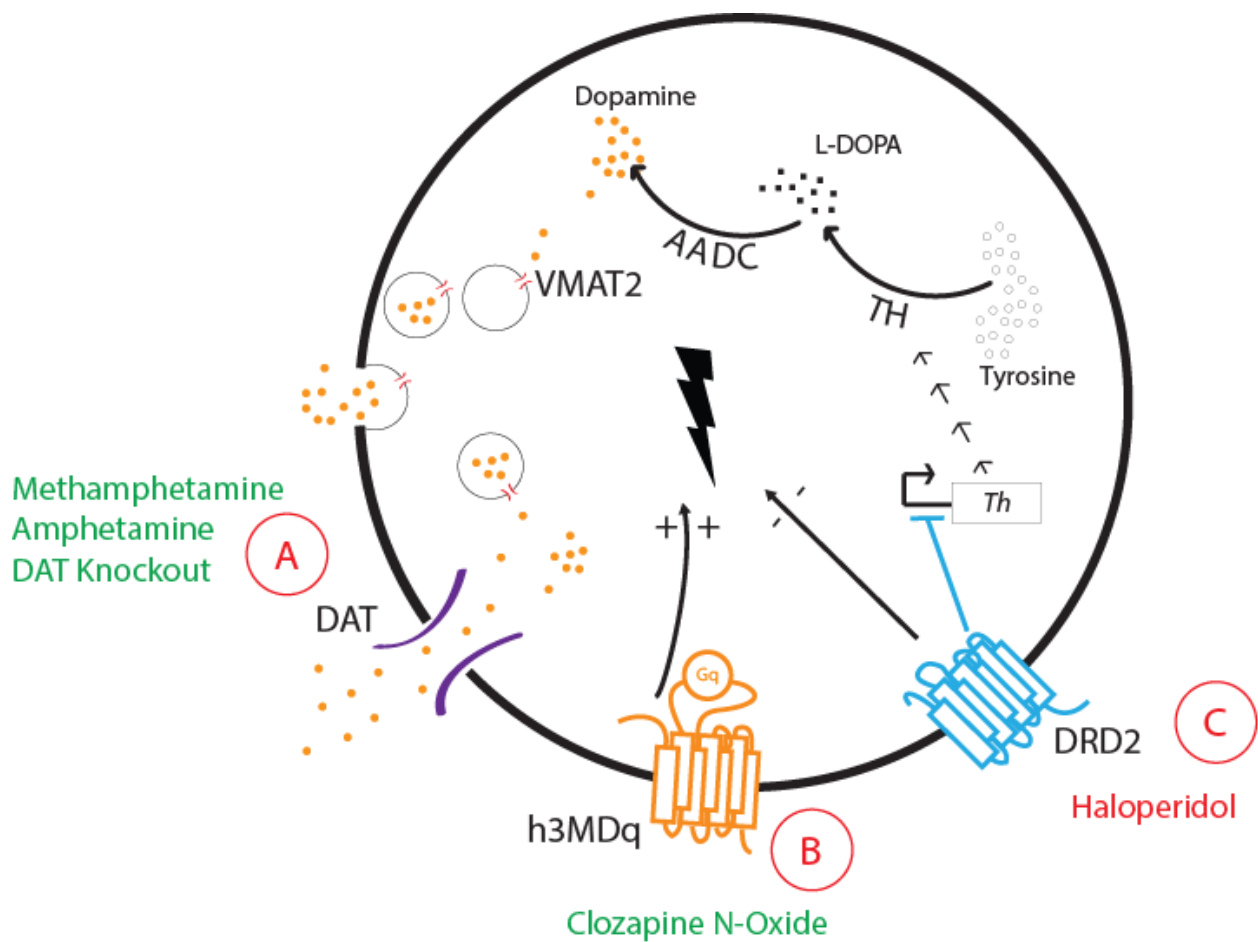


Figure 21: Recapitulation of evidence for a dopaminergic ultradian oscillator (DUO). **(A)** Manipulating dopamine transporter (DAT) activity with chronic methamphetamine, ad libitum amphetamines, or genetic knockout (*Slc6a3*^{-/-}) increases the endogenous period of the DUO. **(B)** Chemogenetic activation of neuronal firing using virally expressed DREADD (h3MDq), and the non-endogenous ligand clozapine-N-oxide activation also increased the period. **(C)** Inverse-agonist action of Haloperidol on the dopamine receptor D2 (DRD2) receptor decreased DUO period in both naïve and methamphetamine treated mice.

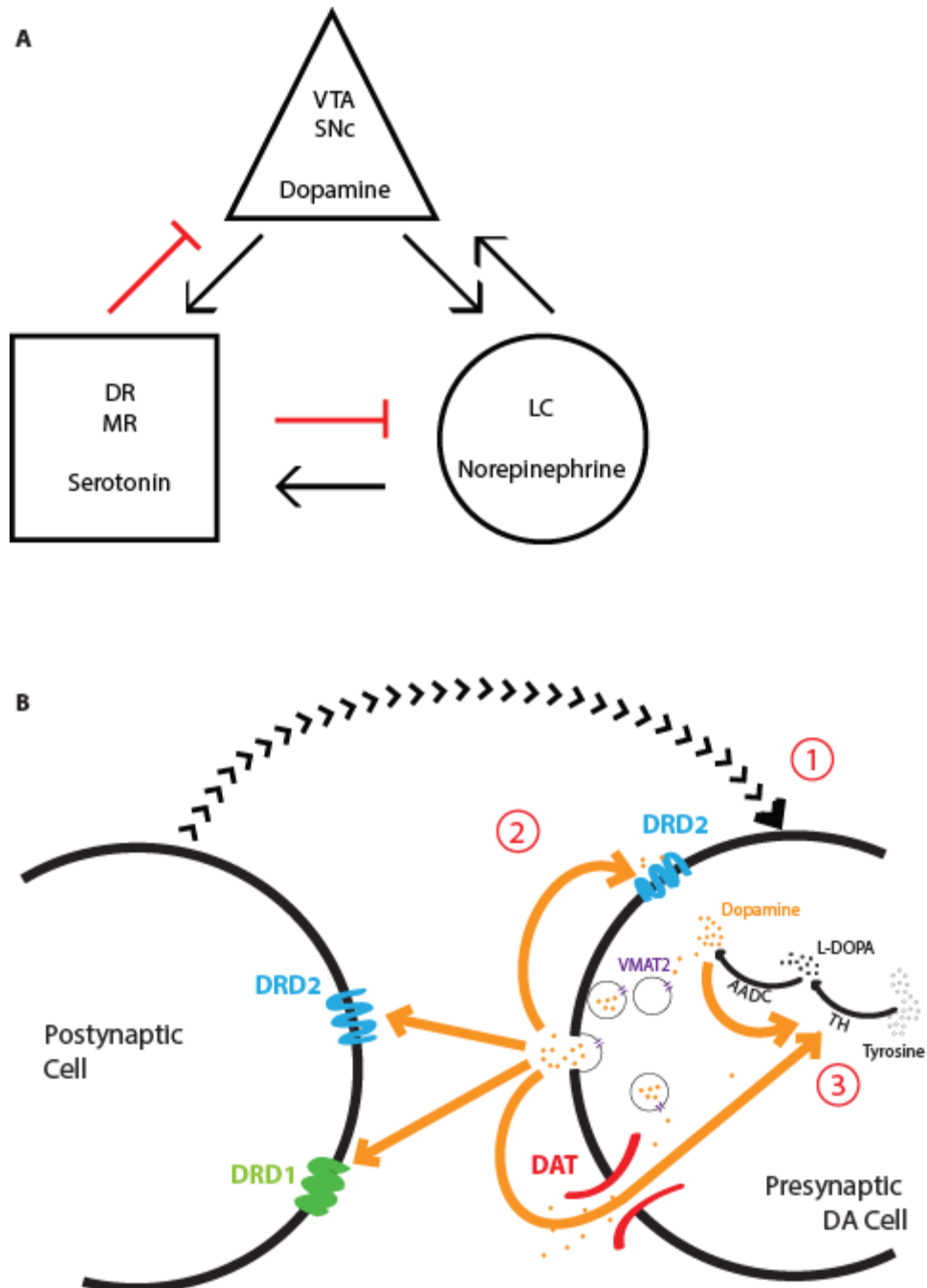


Figure 22: Putative feedback loops of the dopaminergic ultradian oscillator. **(A)** Reciprocal connectivity between major monoaminergic brainstem nuclei resembles a neuroanatomical feed-forward/feed-back loop which may be critical for DUO rhythmicity. **(B)** Three levels of putative feedback loops exist within the DUO at the extrasynaptic level (1), the extracellular level (2), and the intracellular level (3). Abbreviations: AADC, amino acid decarboxylase; DAT, dopamine transporter; DR, dorsal raphe; DRD1, dopamine receptor D1; DRD2, dopamine receptor D2; LC, locus coeruleus; L-DOPA, levodopa; MR, median raphe; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2; VTA, ventral tegmental area.

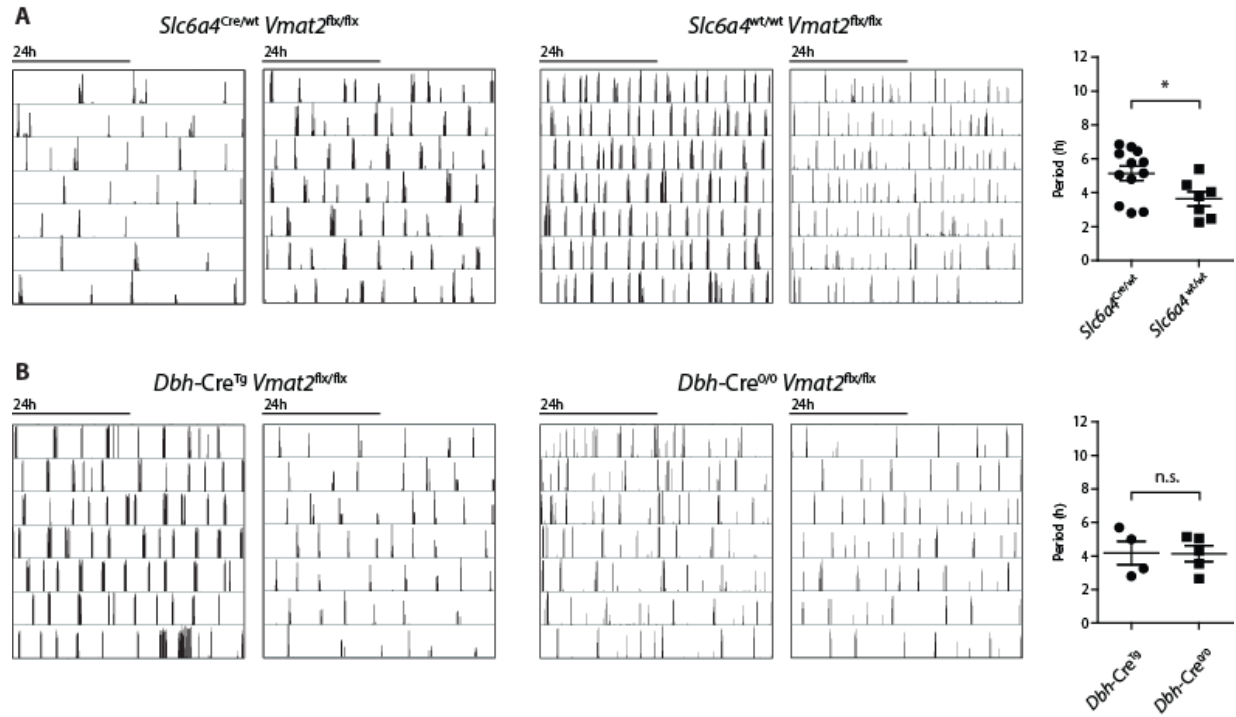


Figure 23: The monoaminergic neuroanatomical feedforward feedback loops are not critical for DUO rhythm generation. (A) SCN lesion of *Slc6a4*^{Cre/wt} *Vmat2*^{flx/flx} mice reveals a slight but significant increase of ultradian period as compared to wildtype littermates (mean ± SEM, *Slc6a4*^{Cre} N=12; *Slc6a4*^{wt}; N=7; $t_{17}=2.337$, $*P<0.05$, unpaired t-test). (B) No such period differences were observed in *Dbh-Cre*^{Tg} *Vmat2*^{flx/flx} (mean ± SEM, *Dbh-Cre*^{Tg} N=4; *Dbh-Cre*^{0/0}; N=5; $t_7=0.5868$, $P=0.9548$, unpaired t-test). Importantly, rhythmicity was preserved in both lines of mice strongly suggesting that a critical feedback loops does not rely upon NFFLs in the reciprocal monoaminergic circuitry.

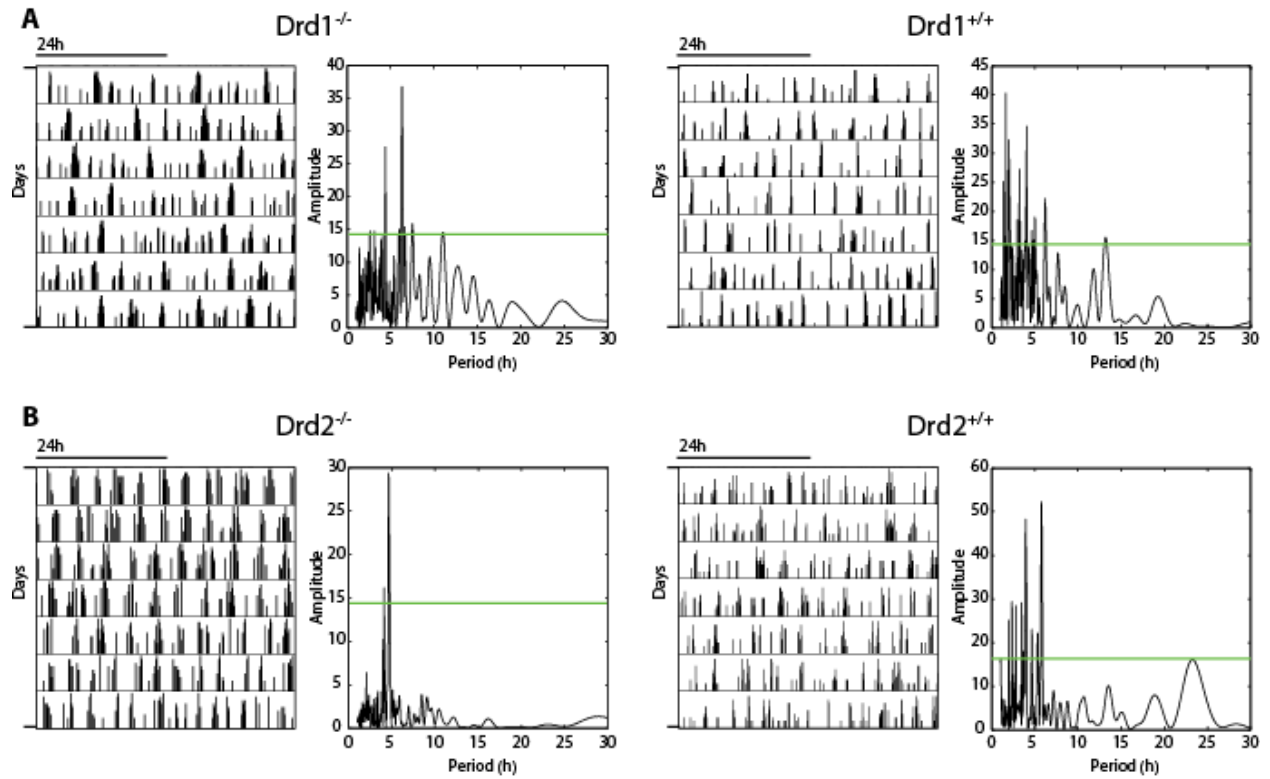


Figure 24: Dopamine receptor knockout mice do not show any qualitative differences in ultradian rhythmicity. **(A)** *Drd1*^{-/-} have regular patterns of ultradian rhythmicity as demonstrated by the corresponding Lomb-Scargle periodogram (immediate right of actogram) which are qualitatively indistinguishable from wildtype littermates (far right). **(B)** Similarly, *Drd2*^{-/-} mice are also unperturbed. Green lines in the periodograms indicates the confidence threshold for rhythmicity ($\alpha=0.01$).

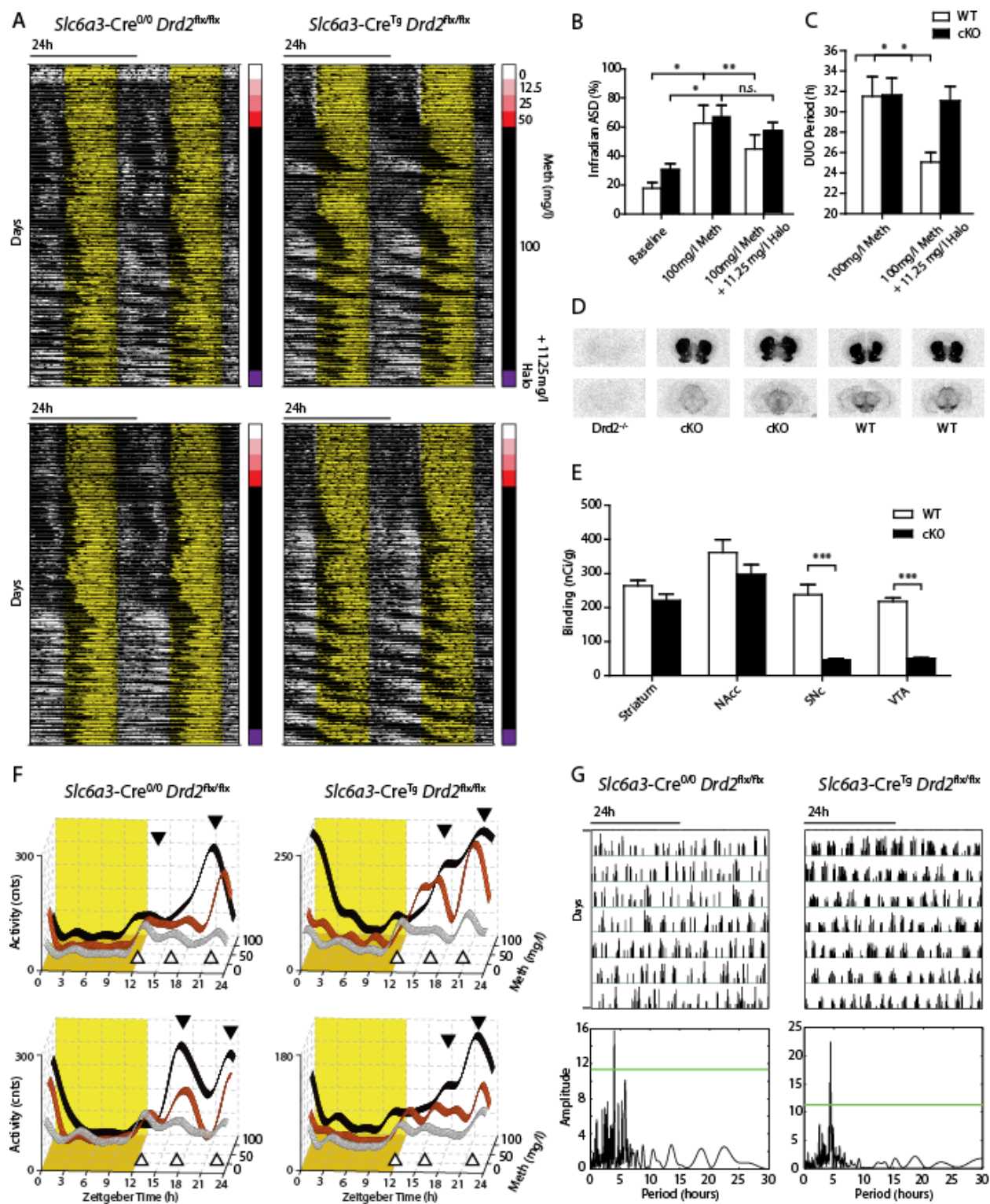


Figure 25: Extracellular feedback onto DRD2 autoreceptors is period modulating under challenged conditions and explains the effect of Haloperidol in DUO period shortening but is not critical for DUO rhythm generation *per se*. **(A)** Actograms of body temperature fluctuations in *Slc6a3-CreTg Drd2flx/flx* mice (right) and their wildtype littermates (left) with color coded legend indicating drug concentration provided ad libitum in the drinking water. **(B)** Comparisons of infradian spectral density (ASD) revealed a significant main effect of drug but not genotype and post-hoc testing revealed a significant decrease in ASD after haloperidol treatment but only in wildtype mice (mean $\pm$ SEM, *Slc6a3-CreTg* N=7; *Sert^{wt}*; N=5; Drug $F_{1,10}$ =14.72, Genotype $F_{1,10}$ =1.38, * P <0.05, ** P <0.01, Repeated Measures ANOVA with post-hoc bonferroni corrected t-test). **(C)** Lomb-Scargle period analysis of methamphetamine treatment in *Drd2* autoreceptor knockout mice before and after cocommitant treatment with haloperidol confirms that DUO period lengthening occurs in these mice but that the period shortening effects of haloperidol act through the *Drd2* autoreceptor (mean $\pm$ SEM, *Slc6a3-CreTg* N=7; *Sert^{wt}*; N=5; Drug $F_{1,10}$ =6.17, Genotype $F_{1,10}$ =3.224, * P <0.05, post-hoc bonferroni corrected t-test). **(D)** Representative images of brain slices showing striatum (top) and ventral midbrain (bottom) for *Drd2^{-/-}* control, *Drd2* conditional knockout, and WT mice demonstrating a marked lack of signal in the ventral midbrain. **(E)** Quantification of the regions of interest extracted from at least two slices per region demonstrate an interaction between region and genotype with a massive reduction in the SNc and VTA of conditional knockout mice (mean $\pm$ SEM, N=5; Interaction $F_{8,24}$ =10.85, *** P <0.001, post-hoc bonferroni corrected t-test). **(F)** Averaged daily locomotor activity of individual mice at different Meth concentrations derived from the time-span indicated by coloured bars next to the representative actogram (A) and subjected to Butterworth filtering. The three night-time activity peaks before treatment (white triangles), transform to 2 peaks after exposure to the highest concentration (black triangles). There are no apparent differences between genotypes as confirmed by analysis of body temperature rhythms (B). **(G)** SCN lesion reveals no qualitative genotypic differences in unchallenged DUO rhythmicity as assessed by locomotor actogram (top) and periodogram analysis (bottom) suggesting that the dopamine D2 autoreceptor are not critical for DUO oscillations to occur.

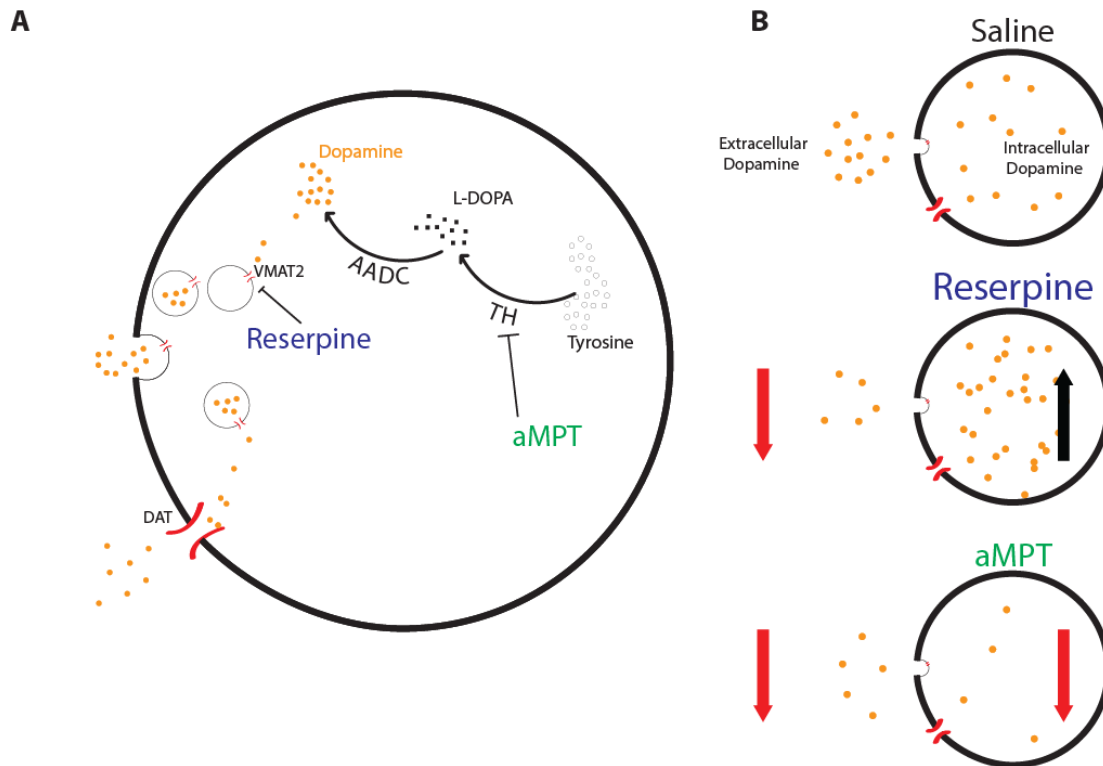


Figure 26: Pharmacological manipulations of dopamine synthesis and release have differential effects on intracellular dopamine levels. **(A)** An intracellular schematic demonstrating the effects of pharmacological manipulation with reserpine acting to reduce vesicular packaging via VMAT2 inhibition and alpha-methyl-p-tyrosine inactivating tyrosine hydroxylase and hence reducing dopamine production. **(B)** The net effects of manipulations on extracellular and intracellular dopamine concentrations demonstrates the dissociation between these two agents, one which can be exploited to determine the location of a putative dopamine feedback loop.

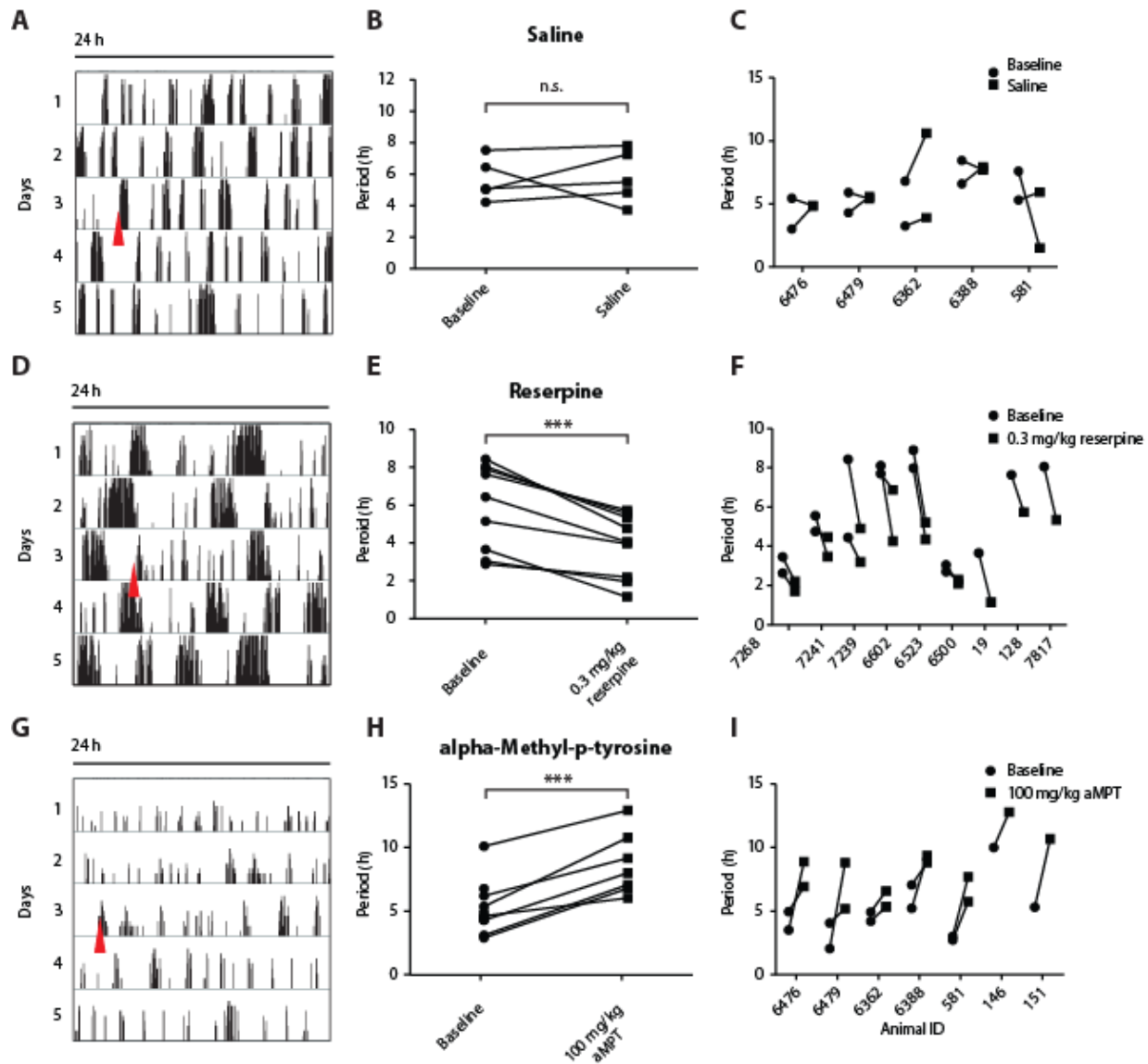


Figure 27: Pharmacological manipulations of vesicular packaging and dopamine synthesis in vivo have differential effects on DUO period. (**A to C**) Locomotor actogram (A) demonstrating locomotion 2 days prior and post intraperitoneal injection of vehicle (09% saline w/v) demonstrates no change in locomotor period as determined by Lomb-Scargle periodogram for 48hs pre and post injection (B, mean $\pm$ SEM, $t_4=0.205$, $P=0.85$, paired t-test). Each trial was repeated twice in the same animals (C) separated by 1 week between injections. (**D to F**) Locomotor actogram (D) demonstrating period shortening response (2d prior and 1 day post) to reserpine (E, mean $\pm$ SEM, $t_4=0.205$, $P=0.85$, paired t-test).with some animals receiving repeated injections (F). (**G to I**) Locomotor actogram (G) demonstrating period lengthening response (2 days prior and 2 days post) to α MPT (H, mean $\pm$ SEM, $t_6=7.408$, $***P<0.0005$, paired t-test).with some animals receiving repeated injections (I).

Chapter 5: Discussion and Concluding Remarks

Discussion:

Is ultradian rhythm generation self-sustained and reliant on intracellular feedback?

Proof of self-sustained oscillations, promoting this oscillator to the role of biological clock, would be best achieved by removing all timing cues, however our lack of knowledge concerning the critical zeitgebers of this new oscillator precludes us from knowing, and hence removing such timing cues, and as such our demonstration must take a less direct approach. Since self-sustained oscillations critically rely on controlled delay of inhibitory feedback, as discussed in the introductory section, the observation of a physiological mechanism for this delay might demonstrate, at least circumstantially, that this oscillator is indeed a biological clock and not just a homeostatic mechanism or hour-glass timer; at least in that absence of direct proof of self-sustainability (with absolutely all zeitgebers are removed). Furthermore, a better understanding of the mechanisms involved may be critical for understanding the intrinsic period of this biological clock, and may provide molecular and genetic targets for pharmacological and behavioural interventions to ameliorate potential disruptions within this oscillator that may underlie a variety of diseases (which will be discussed later). While we have made a significant contribution towards systematically ruling out potentially critical feedback loops, especially those residing outside of the dopamine cells themselves, we would be remiss if we did not discuss some putative intracellular feedback loops which will be tested shortly.

A central candidate with the potential to modulate dopamine signalling in a rhythmic manner is tyrosine hydroxylase (TH), a key enzyme in the dopamine synthesis pathway. Any change in TH biosynthesis, either at transcriptional or even post-translational level might introduce a putative feedback mechanism, taking some prescribed amount of time to replete intracellular dopamine stores to levels capable of feeding back onto the DUO oscillator. In support of this notion the post-doctoral fellow in my lab, Lei Zhu, recently presented evidence of TH activity oscillations, *in vitro*. There are three possible mechanisms for feedback of dopamine upon TH: transcriptionally, post-translationally, and directly at the enzymatic level, via product inhibition. We will now describe these putative mechanisms in more detail.

Currently, there are at least 6 putative transcriptional regulatory elements known within the 5' region (500bp upstream) of the TH gene. The most interesting element, for our purposes, an activator protein-1 (AP-1) functional binding site for the immediate early oncogenes cFos and cJun (378) both of which are expressed in response to electrical input into neurons (for review see 379). In this way, feedback might occur through changes in the electrical state which in turn then affects transcriptional activity leading to alterations in tyrosine hydroxylase levels and thus perhaps dopamine production. Indeed, there are numerous intracellular second messenger signalling pathways that have been identified in neurons which might be operational in DA neurons as well. These are all candidates to inhibit the electrical activity of dopaminergic neurons and thus could have significant impacts on the transcription rates of TH via reduced immediate early gene expression. This mechanism requires recruitment of one or more rounds of transcription and translation and therefore is likely to occur on the timescale observed for the ultradian rest-activity cycle, making it an interesting candidate. To resolve this issue we are currently quantifying TH protein levels using western blotting in *Bmal1*^{-/-} mice at the peak and trough of DUO rhythms. Any oscillation in TH might implicate a transcription/translation mediated feedback mechanism.

Post-translationally, the most prominent effector on dopamine biosynthesis appears to be phospho-activation of TH enzymatic activity with 4 phosphorylation sites ascribed to the TH protein. The most well-characterized is a Serine residue found at amino acid position 40 (pSer40) (380). It can also be considered the most promiscuous phosphorylation site given that several classical second messenger pathways will mediate phosphorylation of this residue including calmodulin-dependent protein kinases (CaMPKII), mitogen activated kinases (MAPK1 & 2), and protein kinases (PKA,B & C) (for review see 379). Reduced phospho-activation caused by a reduction in signalling via any one of these pathways could ultimately introduce inhibitory feedback and a subsequent delay in dopamine biosynthesis, until inhibitory feedback is removed and phospho-activation of a significant proportion of available TH proteins occurred. We are also currently using the same animal models alluded to in the preceding section to test for fluctuations in the various phosphorylation-states of TH which might account for oscillations of enzymatic activity and thus ultradian locomotor rhythm generation.

Finally, there is evidence for an intracellular feedback inhibitory loop of dopamine in form of product inhibition onto TH (for review see 379). TH inhibition by dopamine has been observed to occur at two distinct sites. The first is a high affinity site, only detectable/accessible when Ser40 is

dephosphorylated, leads to drastically increased synthesis rate when dopamine is unbound. Given the affinity of this site it is likely be occupied almost constitutively (381), perhaps acting as a guard against dopamine levels falling too sharply, and responding with strong TH activity to compensate. The second is a low affinity site present in both the phosphorylated and dephosphorylated state of Ser40. Interestingly the K_m constant (an indication of binding affinity) for a required cofactor, tetrahydrobiopterin (BH_4), was low for both TH and Ser40-pTH but increased dramatically (~ 80 -fold) in the presence of dopamine with the slopes of the Eadie Hofstee transformations linear and overlapping, strongly suggesting the Ser40 site is less critical for dopamine's antagonism of BH_4 substrate utilization (381). Importantly, the dissociation constant of this dopamine binding site is ~ 60 nM, which places it into the range of the average cytosolic concentrations of dopamine in midbrain neurons (<100 nM)(382) suggesting that it could easily oscillate around that critical tipping point over time as dopamine concentrations rise and fall according to feedback state. Is it possible that this could happen on the timescales relevant for DUO oscillations? It is possible, but a putative delay mechanism will need to be identified as biochemical feedback of this kind should happen very quickly, on the order of minutes rather than hours, unless of course the second-order product (dopamine) is normally sequestered from TH. In this case the delay could actually be introduced by the time necessary for dopamine to fill the putative sequestration vessel (i.e. synaptic and/or storage vesicles) before it can build up to critical cytosolic concentrations for biochemical feedback. In this way feedback might occur very quickly but only after a protracted amount of time being shuttled into vesicles. Quantal release of dopamine is known to rely on vesicular monoamine transporters (VMAT) to control vesicular packaging and hence dopaminergic signalling tone (for review see 383) with VMAT2 considered the neuronal form. There are several lines of evidence implicating modulation of VMAT2 in control of dopamine release. DAT inhibition by methylphenidate administration *in vivo* increases VMAT2 levels in purified striatal synaptic vesicles and this was correlated with increased vesicular DA uptake (384). Perhaps we could consider vesicular packaging as a mechanism for dopamine sequestration. It is certainly consistent with the widely held notion of granular storage pools of dopamine, and it could directly explain the effects of reserpine, A VMAT2 inhibitor, on DUO period shortening by decreasing the effective size of the dopamine sink that the vesicles represent in this case and leading to faster cytosolic feedback. Unfortunately, genetic models of VMAT2 ablation in dopamine cells produce animals which do not survive for more than a couple days postnatally but this might be enough time to investigate this hypothesis *in vitro* using primary neuronal cultures of *Slc6a3*-Cre^{Tg} *VMAT2*^{flx/flx} mice.

Regardless of the source of the delay mechanism it may be possible to test product inhibition by manipulating BH₄ cofactor availability (385, 386), whose TH binding pocket overlaps with that of DA, or an inhibitor of the rate limiting enzyme of BH₄ biosynthesis, GTP cyclohydrolase 1 (387). In order to properly control for oscillatory behaviour one could chronically infuse these agents i.c.v. into *Bmalt*^{-/-} mice and look for period changes of the ultradian oscillations. One would expect increased BH₄ to outcompete inhibitory dopamine tone, significantly lengthening the time it would take for feedback to occur or possibly even abrogating dopamine feedback altogether. Interestingly, if the dopamine storage pools do represent a critical delay, then the use of a GTP cyclohydrolase inhibitor (increasing feedback sensitivity) should not appreciably alter endogenous DUO rhythms. While neither of these compounds are specific to the dopaminergic system, our data presented here for the other monoaminergic populations suggests that it should not be an issue and any major effects observed could be specifically attributed to dopamine production. Another, more specific method for testing the necessity of TH, but not necessarily direct feedback onto it, would be to use a genetic model specifically lacking TH in dopamine cells. Interestingly, there are several lines of mice that model TH loss including DAT-Cre^{Tg} TH^{flx/flx} mice from Dr. Darvas' group (388) as well as the dopamine deficient (DD) mice from Dr. Palmiter's group (389) which we are currently acquiring. In both cases, survival depends on a single daily injection of L-DOPA to drive dopamine production but in our case we will simultaneously lesion their SCNs and implant chronic infusion pumps for constant delivery, and hence constant dopamine production. If any feedback regulation of TH, be it transcriptional, post-translational, or even biochemical product inhibition is critically required for DUO oscillations then these mice should not show ultradian rhythms of behaviour in constant darkness. Alternatively, observing ultradian rhythms in these mice would suggest that DUO oscillations occur via some other feedback mechanism that does not critically rely on TH.

In vitro models of DUO rhythmicity to better probe biochemical feedback

Intracellular feedback loops open the potential for modeling DUO oscillations *in vitro* and this is an exciting proposition for several reasons including the ability to quickly manipulate and mutate genes of interest using a variety of tools that are prohibitively expensive or slow *in vivo*. While CRISPR and other gene editing technologies are quickly lowering the bar for entry into creating mouse models of genetic perturbations, *in vitro* proof of principles are still an excellent entryway allowing for fast and inexpensive probing of molecular targets of the DUO.

Due to their ease in handling over primary cells, a first attempt to detect intracellular oscillations in DA producing cells, might use the neuroblastoma cell line MN9D (390). Importantly, we detected intracellular DA to fluctuate rhythmically, based on HPLC analysis of cellular extracts and immunohistochemical visualization of intracellular DA. Alternatively, the use of cultured primary DA neurons (391) especially those purified by fluorescent auto cell sorting (FACS)(392), will largely eliminate the complications surrounding targets of dopamine biosynthesis and degradation as they share common pathways with all monoamines. The great lengths necessary thus far to account for these other cell types and neuronal pathways *in vivo*, especially with respect to pharmacology but also with some of our genetic manipulations, can be handily avoided. Purified cell lines also increase the opportunity for homogenous transcriptional expression and regulation, drastically reducing the noise in the system and increasing detectability of specific gene products and protein interactions. In particular, the close proximity of dopaminergic ventral tegmental, substantia nigral, and ventral periaqueductal neurons in the mouse brain preclude the use of many techniques to clearly distinguish the unique roles of these dopaminergic subpopulations in DUO function.

Key drawbacks, or at least challenges, of cellular modeling *in vitro* will be the measurement of DUO output. Based on the data presented within this thesis we currently have two major methods for assessing DUO period, amplitude, and phase: locomotor activity and extracellular dopamine tone. While locomotor activity is clearly unobservable in the dish even extracellular dopamine may be an issue. There is a serious concern that washout of any dopamine released might occur because the typical extracellular space of a dopamine neuron is replaced by milliliters of growth medium. This could complicate measurements of DUO oscillations by HPLC of the effluent, but worse it could negatively impact on the ability of these cells to reuptake dopamine thereby altering feedback parameters, halting them completely in a worst-case scenario. While some groups have reported the ability to perform single-cell patch voltammetry in slice preparations, unfortunately the intracellular dopamine levels in these instances fall below the detection limit of the devices, at least in midbrain dopamine cells although not in peripheral chromaffin cells (382). Importantly however, these studies also suffer from the pitfalls of extracellular dopamine washout, perhaps more so, as these are acute slices perfused with aCSF. This issue might be resolved by pre-treating dopamine cells with reserpine, thereby preventing synaptic release. Our *in vivo*, and some preliminary *in vitro* work, suggest this might be an effective strategy. In our own preliminary attempts using MN9D cells (369) we were able to detect intracellular DA upon differentiation into neurons by HPLC of pooled

cytosolic lysate and immunocytochemically using glutaraldehyde-conjugated-DA antibodies (370) but only after 30-minutes pretreatment with reserpine. Unfortunately, as dopamine is a small molecule, genetic manipulations to generate fluorescent fusion proteins is not amenable. Neither is bioluminescence, at least until a suitable transcriptional oscillation is identified in these cells. Alternatively, if TH is a critical target at the level of post-translation or product inhibition then perhaps a fluorescent resonance energy transfer (FRET) or BRET (using bioluminescence) approach might be undertaken, although that will require a very serious commitment of both time and resources to generate. While we feel the results could ultimately be worth the reward, there is certainly not enough evidence, nor a suitable target for such an undertaking at this time. That a molecule like dopamine has been studied for so long without the development of a tool to visualize or measure its production, and especially cytosolic concentrations, in a manner suitable for our purposes suggests that either it is a very difficult task indeed, or that sufficient motivation was simply never provided. Certainly the advances in voltammetry and amperometry have all geared towards highly time-resolved measurement of dopamine release in vivo with much less attention paid to biochemical regulation of its production and degradation within these cells. There was certainly never any desire to observe network interactions of cytosolic dopamine regulation and certainly not on the timescale of minutes to hours, although hopefully we have provided some evidence that may lead to a change of focus in the study of DA's role as neuromodulator .

Potential Interaction between the DUO and the circadian system

We propose that the daily pattern of arousal is regulated by two oscillatory systems in concert: the DUO and the SCN circadian pacemaker (Figure 20A). While the SCN pacemaker period is designed to be invariable, set at a cycle length of almost exactly 24hrs, the DUO can adopt periods from a few hours to >100 hours as a consequence of changes in dopamine tone. Interestingly, there is disparate evidence observed throughout our studies, but also reported elsewhere, which suggest that instead of acting as completely independent entities, whose outputs are integrated downstream, that there is crosstalk directly between these two biological rhythm generators.

DUO rhythms are clearly impacted by the circadian system as endogenous duo rhythms often occupy harmonics of the circadian system in intact (Figure 12) mice. Interestingly, the shape of the distributions of the CWT period averages, with a smooth sigmoid distribution in *Bmal1^{-/-}* and

discontinuities at non-harmonic intervals in SCN_x mice (Figure 8), hints at a distinct contribution of circadian clocks within the DUO itself. We wonder if this would have been even more pronounced by reporting the median periods from the CWTs rather than period averages. This is supported by the work of many others, demonstrating the circadian regulation of dopamine production and release (269, 308, 309, 393-397). Critically however, many of these experimental paradigms potentially suffer from under-sampling with only 2 to 6 timepoints per day, certainly enough to demonstrate a circadian rhythm but aliasing (due to under-sampling) of DUO rhythms are distinctly likely. Ultimately, with higher sampling frequency many of these supposed ‘circadian’ oscillations may actually be occurring in the ultradian range and therefore follow DUO and not circadian regulation. Unfortunately it will not be easy to redo all of these studies with a higher sampling rate as this would be too onerous, require too many samples, multiplying the work greatly. Instead we suggest the use of *Bmal1*^{-/-} mice in constant far-red lighting as we did for our own microdialysis experiments for the determination of extracellular DA levels. As the sampling will be dependent only on the interval of the ultradian cycle, the sampling time span can be greatly reduced as the ‘confound’ of the circadian clock does not need to be taken into consideration which would require longer sampling times (e.g., 24 hours). Another potential caveat to consider is the relative flexibility of the DUO with respect to circadian phase. In our own experiments DUO rhythms are only observable at the behavioural level when they are averaged over multiple days within the same individual. Indeed averaging across mice tends to obscure the three peaks at night as the differences in peak timing between mice tend to cancel or otherwise interfere with each other (data not shown). There is a distinct possibility in other studies, like those performing *in vivo* microdialysis (308, 398), that this is similarly obscuring their detection of ultradian rhythmicity. With these two suggestions for experimental design we firmly believe that the distinct contributions of ultradian and circadian regulation of dopamine production, release, and degradation might be better resolved.

Further evidence of SCN→DUO interactions is the relative coordination of the DUO to SCN signals in DATKO mice in constant darkness with bending of the DUO onsets at or transitions through the circadian oscillations (Figure 16D). This has also been reported for methamphetamine treated mice in both DD (143) and LD (399). Furthermore when they near the circadian signal they ‘discontinue’, i.e. DUO rhythms never show onsets late in the circadian cycle, we interpret this as them quickly phase-delaying to synchronize with the next circadian onset rather than just being masked, since the next free-run often starts days before it would be predicted from

drawing a straight line through the duo onsets (Figure 16G and in (144)) and this is also apparent in the actograms of body temperature (Figure 25A). Finally, methamphetamine lengthened DUO rhythms snap back to precisely 24 hours after haloperidol treatment of circadian intact mice (Figure 13E,F and Figure 25A,C), but this does not occur in similarly treated *Bmal1*^{-/-} mice (Figure 13C,D). Furthermore circadian intact mice lacking the dopamine transporter appear to adopt 4 hours rhythms that are phase locked to the circadian system (or at the very least the L:D cycle) after the highest dose of haloperidol treatment (Figure 13G).

Conversely the circadian system also appears to be altered by the DUO phase and period. DATKO circadian-intact mice show relative coordination of the circadian free-running signal in DD. Scalloping/wobble of circadian component suggests that the DUO can pull the SCN to longer rhythms when it is itself free-running (Figure 16D). This is also corroborated by the phase delay of the onsets of the circadian system upon DUO free-run under LD. Notice that the onsets of activity occur after lights off. This is atypical for mice where light is usually masking locomotor onset and hence activity begins immediately before or at the light to dark transition (Figure 16A vs G) and this has also been reported by others (144). There is a plethora of literature suggesting that dopamine induced arousal has the ability to alter circadian behaviour in rodents (reviewed in 400) and flies (401). Interestingly the effects of dopamine on nighttime arousal in drosophila act directly through *Cryptochrome*, a canonical clock gene (402). The function of dopamine D1 receptors expression in the foetal SCN is attributed to maternal synchronization of the rat pups in utero (for review see 403) with TH-positive fibres detected in neonatal rodents (404) while the initial sensitivity to dopamine signalling rapidly declines after birth (405). However the role of these receptors in adulthood, with mRNA expression still present throughout the adult mouse, primate and post-mortem human SCN? (350, 406), is currently unknown. Interestingly mice and primates lacking nigro-striatal dopamine show severely impaired circadian regulation of locomotor rhythms (407, 408) mimicking the effects of TH mutations in flies (401). Several groups report that drugs of abuse produce drastic changes in light entrainment and resetting responses to light pulses (409, 410) and methylphenidate alters behavioural and SCN electrical and molecular rhythms (411, 412). Importantly, these drugs are not strictly dopaminergic and indeed it seems as if the majority of these effects are attributable to serotonin reuptake blockade (413, 414) yet the contribution of dopaminergic effects have never been ruled out. Just this year, optogenetic stimulation of DRD1-expressing neurons within the SCN has been shown to strongly resets circadian molecular and behavioural rhythms (415) but these mice

used a congenital Cre-loxp system as ‘a proof of principle’ and so the relative contribution of adult-specific DRD1 expressing cells is unresolved. Perhaps the injection of viral-mediated DREADDs or optogenetic channels into adult mice might resolve this longstanding question.

Relevance of the DUO to psychopathology and potential impact

Biological clocks have evolved in mammals as mechanisms to help anticipate regularly occurring changes in the environment such as sun rise or food availability. Lessons from studying the circadian system over the past half century teach us that these clocks are so crucial for survival, and so engrained in our biology, that clock dysfunction can have debilitating consequences for physiological and mental health. While the underlying functional significance of ultradian rhythms in dopamine and the rest-activity cycle are still unknown, there are a number of potential clinical implications for disturbances to either the amplitude or the period of these oscillations. Our findings suggest that distinct daily activity pattern aberrations associated with certain psychiatric conditions might stem from DUO dysfunction.

For example, numerous lines of evidence suggest that schizophrenia is associated with hyperdopaminergia and these form the intellectual basis for the putative “dopamine hypothesis”. At the heart of this theory is the seminal work of Carlsson and colleagues who reported that early antipsychotic agents acted by increasing the metabolism of dopamine in animals (416). Furthermore, the antipsychotic haloperidol acts as antagonists or inverse agonists of the D2 receptor, leading to a reduction in dopaminergic signalling (417). This action could be interpreted as disrupting hyperdopaminergic signalling that might be at the core at the dysregulation leading to schizophrenia. Similarly, abuse of dopamine enhancers such as cocaine and amphetamines can cause psychotic states and schizophrenics are considered supersensitive to these drugs (418), attributed in part to changes in the conformational state and/or expression of D2 receptors (419-421). Importantly, schizophrenia is characterized by specific disturbances of locomotor activity and the sleep-wake cycle (277, 422, 423) with some subjects showing patterns of behaviour that are strikingly similar to those observed in DAT^{-/-} or methamphetamine-treated wild-type mice (Figure 28A).

Bipolar disorder is similarly associated with altered rest-arousal cycles (281, 283) and hyperdopaminergia has been linked to the disease (280). There is also a strong correlation between altered 24 hour patterns of behaviour and affective state in sufferers (424) These 24-hr sleep:wake cycle impairments are currently attributed to a dysfunction of the circadian system (309, 425-428).

Our data now provides an alternative rationale: the rest-arousal pattern aberrations are due to a dysfunctional DUO with an altered temporal pattern of dopamine tone. Accordingly, high extracellular dopamine tone with correspondent low cytosolic dopamine levels, leads to a lengthening of the DUO resulting in arousal rhythms that are dissociated from the circadian system and a sleep:wake pattern that is greatly perturbed. A lengthen DUO may entrain at circadian frequencies (Figure 28B, top left) or at a period longer than 24-hr causing the DUO to “free-run” (Figure 28B, top right) and resulting in a starkly abnormal daily rest:arousal patterning that changes over time. Importantly these changes are not random but follow a pattern of successive shifts in the daily sleep/activity onsets and may phase lock again at 48hr, a frequency harmonic to the entrained circadian cycle. In this situation, the extra arousal is produced only every second day, i.e., the DUO shows so called ‘circabidian’ behaviour (Figure 28B, bottom right). Most prominently, this 48 hour rapid cycling is seen in some bipolar subjects (283, 284), where manic episodes precisely occur every

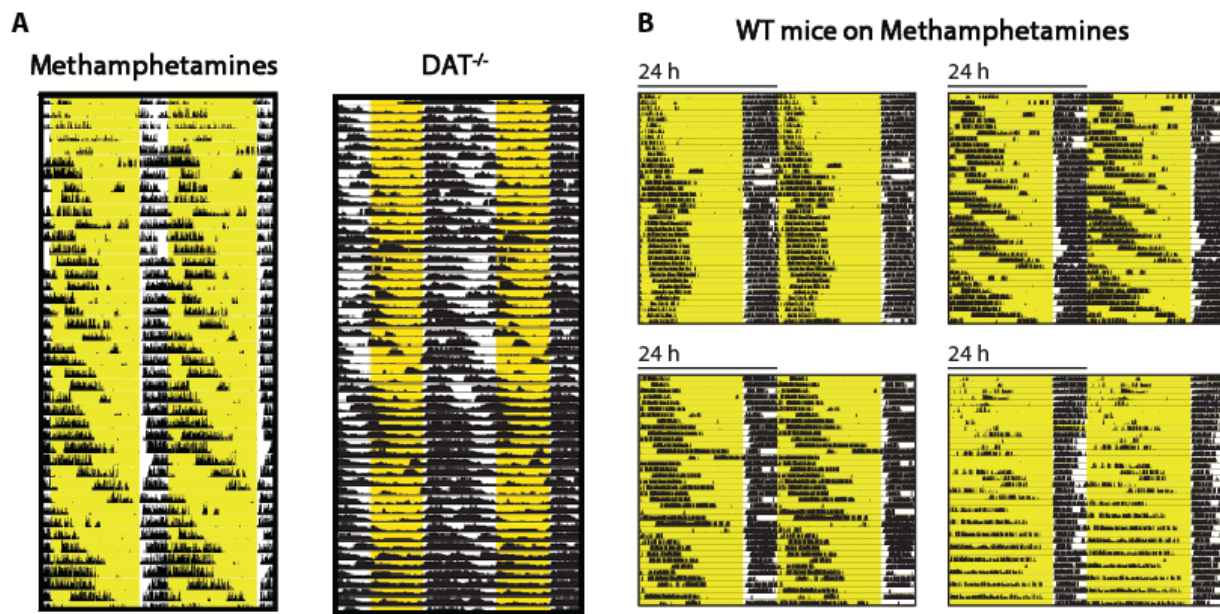


Figure 28: Patterns of locomotion are composed of the output of at least two distinct oscillators: the SCN and the DUO. (A) Free-running DUO rhythms in methamphetamine treated and dopamine transporter knockout mice with intact circadian systems under a light:dark cycle demonstrate striking similarities to locomotor patterns from some schizophrenic subjects (Please see Figure 3H,I in 277) and the sleep-wake patterns of bipolar subjects (Please see sleep recordings for the years 1993 and 1994 in Figure 1 of 284). (B) Chronic ad libitum methamphetamine in the drinking water (100mg/L) of wildtype mice on a pure C57BL6/J background results in a wide variety of DUO periods from 24-h phase delayed rhythmicity (top left) all the way to 48-h cycling (bottom right).

second day, could be explained by the DUO oscillating with a period of 48 hours resulting in extended episodes of arousal every second day as observed in mice. Interestingly, polymorphisms in a key dopamine degrading enzyme was found strongly enriched in subjects exhibiting ultra-ultra-rapid cycling bipolar disorder (429), i.e. those with the longest free-running DUO period, but not subject with less frequent bipolar episodes.

Similarly, the distinct activity pattern seen in schizophrenic subjects (277) might be equally attributed to DUO period lengthening. The fact that both diseases have been associated with heightened dopamine tone further corroborates our hypothesis of a dysregulated DUO during manic and schizophrenic episodes. Our findings may provide a unifying principle for several major mental disorders with altered dopamine tone, especially those with a known temporal component. In general, increased amplitudes would conceivably create sleep disturbances by increasing arousal during normal resting hours, while decreased amplitudes would lower physiological arousal in general, with implications for depressive-like symptoms. Alterations in the period of these oscillations on the other hand create the potential for desynchrony with other physiological processes, most notably the circadian control of sleep-wake cycles, metabolism, and cognitive function. These results could have implications for neurodegenerative diseases like Parkinson's, whereby neurotoxicity or redox state could reach unsustainable levels without proper timing of feedback controlling arousal. Conversely, destruction of the dopaminergic system, as occurs during the progression of Parkinson's disease could produce notable alterations in arousal, including hypersomnia and sudden onset sleep attacks (430) as well as fragmentation of ambulatory activity (431). Manic depression is also a potential candidate for DUO dysregulation, whereby free-running infradian oscillations (as observed in the DATKOs) would gradually transition in and out of phase with the circadian system over a period of weeks or even months, creating sustained and alternating periods of both hyper- and hypo-arousal which would themselves oscillate on a timescale of weeks to months. Similarly, approximately 24-hr oscillations in the dopaminergic system could also cause positive symptoms of schizophrenia if these oscillations were phase locked but anti-phasic to the circadian system. By selectively increasing ascending dopaminergic signalling within the brain, while the circadian system simultaneously suppresses other regions, and vice-versa, in a time of day dependent manner, one might envision wildly pathological interactions within the networks controlling consciousness and perception. Restless leg syndrome, is another candidate for such

dysfunction as descending dopaminergic projections might be hyperactive during normal resting hours when they would be expected to be suppressed (432).

Final Remarks:

The study of a newly discovered timing mechanism within the dopaminergic system, a system which is critically important for human health as suggested by its potential involvement in a wide range of psychopathologies and other disorders, has significant potential to contribute to our understanding of the neurobiology underlying these disease states. Here we investigated the mechanism that contribute to the proper functioning of two such biological clocks, identifying potential molecular and cellular targets for treatment of malfunctioning systems, and in the second case developed a mouse model which can, moving forward, help us to better understand the health consequences when the DUO goes awry. In the future, the use of this animal model for the development of easily measured diagnostic criteria, so called biomarkers, might allow us to non-invasively observe changes in the dopaminergic system, and further contribute to our ability to scientifically study these diseases, to track their progress after medical or psychological intervention, and possibly even to predict their appearance in an individual before other readily observable outward signs.

We sincerely hope the discovery of the DUO represents a true breakthrough in our understanding of rest-arousal/sleep-wake regulation. This novel insight into the biology of mental illness may pave the way to new treatment strategies aimed at “reigning in” a free-running DUO by using appropriately timed drugs, light, and/or behavioural interventions targeting the dopamine system. We also hope that it might more widely expose psychiatrists and clinicians to a potentially crucial biomarker for mental illness: actigraphy – an easily employed measure to detect, monitor, and perhaps even predict psychiatric disorders in humans and animal models alike.

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Technical Addendum:

Cre-mediated ablation and resurrection of BMAL1

While we were able to successfully ablate *Bmal1* expression from VCKO mice, we did not achieve acceptable results for ACKO mice. This is paradoxical in some ways as the transgenic insertion of Cre, as opposed to an internal ribosomal entry site knockin (IRES) generally has the potential to yield much higher rates of expression due to potential multisite landing and therefore higher potentials for transactivation of mRNA transcription (194). This may be due to higher endogenous levels of transcriptional activity at the *Vip* promoter as opposed to *Avp* promoter although this has never been quantified directly to our knowledge. Clearly the *Avp*-Cre line was capable of producing sufficient cre-recombinase in AVP+ cells in order to express the genetically encoded fluorescent indicator TdTOMATO, a knock in at the *Rosa26* locus, but not abolish BMAL1 expression in *Bmal1^{flx/flx}* or even *Bmal1^{flx/-}* in the SCN, suggesting a difference in the accessibility of the *Bmal1* genomic locus or more specifically, exon 8 of *Bmal1* to Cre-recombinase. We can rule out a universal issue with the *Bmal1^{flx/flx}* line as this has been successfully used to ablate BMAL1 by employing different Cre drivers from various cell types in the periphery (74, 199), the brain (202), and even the SCN (135, 201) including our own success in VIP+ cells, although in the case of *Synaptotagmin10* they did require two copies of the Cre allele and hemizygous flx-*Bmal1* for efficient recombination in the SCN, as required here for our VCKO mice. Taken together this argues strongly for a reduced accessibility to the *Bmal1* loxP sites specifically within the SCN. This could be due to a variety of factors including the increased presence of transcriptional machinery around the clock acting on this gene, interfering with Cre-recombinase activity. This idea supported by the high amplitude expression of the mRNA and the protein product of *Bmal1* specifically within the SCN. Of note, a very recent study was published where the authors successfully used *Avp*-Cre^{Tg} mice to excise *Bmal1* however it is clear that the iCre line (improved Cre-recombinase) was generated in house (196) and indeed not the same one we obtained from the Gensat project, suggesting that we lost the genetic lottery in this regard. This highlights the need, at all times, to properly validate recombination efficiency whenever using a newly generated Cre line, even if the floxed line has previously been successfully used in other contexts.

This naturally leads to a discussion of our inability to resurrect *Bmal1* specifically within the AS mice. At first, it could be considered that the same issues are at play as above, with the Cre line

simply unable to access the *Bmal1* promoter. However there are several arguments against this being the case. First, the site of our *loxP*-flanked stop-cassette is in a different genomic location within the *Bmal1* locus than the *LoxP* sites of the *Bmal1*^{flx} allele. The second is that the design is very similar to the flox-stopped design employed for TdTomato, which showed efficient recombination in the AVP-Cre mice. Except in that case it is knocked in to the Rosa26 promoter, one of the most transcriptionally active constitutive promoters in the whole mammalian genome, strongly arguing that it is not the accessibility of the *Bmal1* locus which is at fault in this case. Finally, even a reduction in recombination efficiency, as seen in the ACKO, mice still resulted in at least some recombination whereas we did not identify even a single neuron expressing BMAL1 in the SCNs of these mice using both immunofluorescence and a more sensitive DAB immunoprecipitation allowing for massive signal amplification. The only putative explanation we can provide for the failure of the resurrection in these mice is the complete lack of transcriptional activity at the *Avp* promoter in SCN cells congenitally lacking BMAL1 (as is the case for both *Bmal1*^{-/-} and *Bmal1*^{fls/fls} mice). The reasoning is as follows: if activation of the *Avp* promoter relies – either directly or indirectly—on BMAL1 transcriptional activity and these mice never express BMAL1, then they will not produce *Avp* nor can they express Cre-recombinase driven by the exact same promoter sequence, regardless of where it lies in the genome. This complete lack of Cre-recombinase expression would obviously then affect the recombination of our floxed-stop cassette, thereby preventing expression of *Bmal1*. While we can only offer circumstantial evidence at this time, in the form of a complete lack of *Avp* expression as assessed by in situ hybridization (specifically within the SCN and not the nearby PVN or SON, Figure 5C), we are confident in our conclusions based on severely reduced SCN specific *Avp* expression in *Clock*^{-/-} mice reported by several groups (26, 177, 203). Interestingly, the SCNs of *Per1/Per2* double-mutant mice still have quantifiable rhythms of AVP release *ex vivo* (204), strongly suggesting that it is BMAL1 which is strictly required for *Avp* transcription within the SCN, and more specifically BMAL1:CLOCK heterodimers, which are necessary for *Avp* transcriptional rhythmicity in the master circadian pacemaker.

Given some of the issues raised above, it might be tempting to dismiss the lack of behavioural rhythmicity observed in VS mice due to a lack of efficient rescue, whereby there were simply not enough oscillators resurrected in order for the SCN to output what is clearly a coordinated rhythmicity (at least *ex vivo* as observed here). Ultimately we were hoping to address this question definitely by comparing and contrasting any phenotypic differences between AVP+

and VIP+ resurrection. We did address this in some way by quantifying and comparing the number of BMAL1 resurrected cells to the total possible pool of Cre expressing cells and found no significant difference (Figure 5). Furthermore there was absolutely no correlation between the number of resurrected cells and the rhythmic amplitudes observed regardless of the significance of the amplitude. Finally, we know that the SCN output driving locomotion must be a diffusible factor (119) and presumably that factor, if it did come from VIP+ cells, would be VIP itself. There are other neuropeptides, gastrin releasing peptide (134), neuromedin-S (135), and little SAAS (433) which colocalize with VIP+ cells but are not exclusive to them. Of course this diffusible factor need not be peptidergic, but as of yet no other putative small molecules have been implicated in locomotion, nor are there any biogenic amines which are spatially restricted in an appropriate manner (434), making peptidergic control the most likely (435). Since we do not observe any obviously discernable change in VIP protein expression, nor the number of VIP expressing cells in these mice (Figure 3D), we are confident that BMAL1 resurrection in VIP+ cells is complete and it is almost certain that these cells do not output to locomotor centers. Other arguments supporting our claims include historical account in hamsters and rats (436, 437), and our own extensive experience with mice, that even with an extremely low number of SCN cells remaining after electrolytic lesion circadian rhythmicity is often observed and indeed recovered over time. This is confirmed by retained behavioural rhythmicity in a small proportion of *Synaptotagmin10^{Cre/Cre}* *Bmal1^{flx/flx}* mice even though BMAL1 expression is typically very low and generally spatially diffuse (<17%, personal communication). Finally, mice with BMAL1 excision using a dopamine-receptor-1 Cre-driver show absolutely no change in endogenous period, rhythm amplitude, or activity levels (438), despite the verified expression of Cre-recombinase efficiency in more than 90% of both AVP+ and VIP+ cells in this Cre line (415). Even mathematical modeling of the SCN network suggests that as few as twenty-five cells in total, when coupled together, might be sufficient to generate the temporal properties of the entire network (439). That isn't to say that only twenty five are capable of driving locomotor rhythmicity *per se* as they would require output to locomotor centers, only that the temporal precision observed could be accounted for by as few as ~0.25% of the cells in the SCN, much fewer than the ~10% accounted for by the VIP+ population. Interestingly, this theoretical hypothesis is also consistent with the highly synchronous and precise oscillations observed *ex vivo* using bioluminescent imaging (Figure 6) and strongly suggests that VIP+ do not contribute to locomotor control.

Bioluminescent imaging for investigating networks of TTFLs

The use of clock controlled bioluminescent reporters, primarily via genetically specified luciferase protein expression, is almost ubiquitous within circadian biology at this point. However, this only complicates matters for the modern researcher as they try to design new experiments as they must now decide from a plethora of both luciferase drivers and the exact “flavour” of luciferase itself. For our experiments we chose to use mice with ubiquitous *Bmal1*-Luc expression for reasons that will hopefully become clear below. The first ever study using luciferase to visualize circadian rhythms *ex vivo* (173) did so by expressing it driven by the *Per1* promoter. More recently, other variants have been generated utilizing different clock gene promoters including *Per2*-Luc (mammals), *Cry*-Luc (*Drosophila*), *Cab2*-Luc (*Arabidopsis thaliana*), *kaiB*-Luc (cyanobacteria) and even fusion proteins allowing for subcellular localization of clock proteins like PER::luc (*Drosophila*), PER2::Luc (*Mus musculus*) and even phosphoproteins like PKaiBS::Luc (cyanobacteria) just to name a few. Furthermore, there is an ever-growing selection of luciferase proteins to choose from including the traditional firefly gene but also some enhanced versions taken from other organisms like beetles or spectrum-shifted in order to allow for multi-channel bioluminescence or better penetration (exit) of the bioluminescent light allowing recording from deeper structures.

Bmal1-Luciferase, in contrast with *Per1*/*Per2*-Luc or even PER2::Luc, does not possess the same visual impact that accompanies high-amplitude expression with a complete-absence of bioluminescence at trough levels. However, *Bmal1* driven bioluminescence is very easily quantifiable and comparable nonetheless. With the particular optical and CCD optimizations designed into our own bioluminescent microscope (at least over those of the early custom design of Dr. Yamazaki) we could reliably detect bioluminescent rhythms from *Bmal1*-Luc SCNs as shown here but also in peripheral tissue e.g. the pituitary (See Supplemental Figure 1 in 225) and even dissociated *Bmal1*-Luc fibroblasts, used for designing and testing the entire system (217), with little issues of signal to noise preventing detecting of rhythmicity at 48 samples a day. Furthermore, we detected significant oscillations with our system even when sampling the SCN with 15, or 9 minute exposure times (data not shown). Pair this with the 10-fold increased brightness of *Bmal1*-Eluc mice (generated using a much more active beetle luciferase)(440) and we could envision designing a multi-well system with the capacity for simultaneous recording of up to six dishes at once given some motion control hardware and programmable software.

Interestingly, the use of *Bmal1*-luc has at least two compelling advantages to its use. 1) The relatively insulated promoter of *Bmal1*, versus the *Per* promoters for instance, means that any recordings more faithfully recapitulate the transcriptional state of TTFL output given that CLOCK:BMAL1 are the primary transcriptional factors mediating clock controlled genes. The transcriptional activity at this promoter is buffered against transient spikes caused by medium changes (441) and other perturbations in the system which might activate CREB transcriptional activity including GPCR activation and neuronal excitation (27). Furthermore, as highlighted in our own study, the loss of TTFL rhythmicity in cells need not result in a blank slate so to speak. With the *Bmal1* promoter constitutively active and relying on transcriptional repression for rhythmicity, even cells with a clamped oscillator can still be monitored for both health and transcriptional activity throughout the luciferase recording. In this way the use of *Bmal1*-luc provides quantifiable proof of a lack of rhythmicity, as opposed to seeing a completely black image when no oscillator is present. 2) A problem not widely discussed in the literature but certainly encountered by almost every researcher using bioluminescence models, *ex vivo* tissue and *in vitro* cultured cells alike, is one of migration over time. In organotypic preps there is a slow spread and flattening of the cultured tissue over the first week or so. Alternatively, in cultured cells like fibroblasts, cells often migrate and shuffle around the dish over time with some lines moving quite far over short periods of time. The issue isn't the movement itself *per se*, it is the loss of signal at the nadir of Per-Luc oscillations. This can wreak havoc with automatic cell tracking software and human observer tracking alike. Early researchers using bioluminescence *ex vivo* were relegated to individually tracing each cell and attempting to follow it over time, with the end result that not very many cells can be isolated (442, 443). Alternatively, some researchers simply wait for the explant to stabilize, but this is not feasible if the phase response, relative to some *in vivo* stimulation prior to sacrifice, or some other dynamic parameters are to be calculated. This also is not an option for dispersed cell cultures, like fibroblasts, as they will never stabilize. While there are certainly commercially, and even freely (444), available tools that purport to aid in the automation of this task, the reality is that if at any point in the oscillation of the cell the bioluminescent signal falls below the threshold for detection, the entire cell will be lost for the remainder of the recording. This is often the case with the high-amplitude oscillations of *Per1*-Luc and even more so with the PER2::luc fusion protein. On the other hand, the oscillating but still mostly constitutive expression of *Bmal1* transcription means that with the use of *Bmal1*-Luc mice, cells are much more rarely lost by these automated cell segregation and tracking tools.

Animal modeling of DUO dysfunction

In our experiences using hyperdopaminergic mouse models, especially in the context of circadian clock dysfunction, we suffer massive experimental attrition. Mice provided ad libitum methamphetamines must have the dosage slowly raised over many weeks and DATKO mice must be first accustomed to single housing before running wheel access can be provided. Furthermore, it was necessary to provide all of these mice, and their wildtype littermates, ad libitum access to constantly shifting palatable treats like freeze dried worms, peanut butter, and chocolate flavoured treats, in conjunction with more than ample nestlet and bedding material. With this extra husbandry we were able to mostly abrogate attrition, reducing it to about 25% over experimental paradigms often lasting several months.

Furthermore, every effort was made to keep the animals housed in a room and/or cabinets with little to no outside influence. Both sound attenuation and a reduction in personal monitoring helped significantly with our ability to gather stable ultradian rhythms. In many cases, cage changes or even the presence of an observer cause DUO resetting, apparently regardless of the phase of the oscillator (Type 0 resetting), and while this appeared to be true for ultradian rhythms in mice under normal conditions it was especially apparent in either DATKOs or mice provided with methamphetamines.

Taken together, the maintenance and study of these models -at least in our hands- turned out to be particularly expensive and laborious. Furthermore, these models suffer from another challenge, a lack of specificity. In the case of methamphetamines (A schedule II drug), results are confounded by effects on both the serotonin- and norepinephrine-reuptake transporters. In the case of DATKOs, these animals carry the mutation from birth and so might suffer from congenital defects or even survival selection in utero and neonatally. There is also the matter of increased hyperdopaminergia in all dopamine neurons, not only of the midbrain but also of the hypothalamus and brainstem, leading to many off target effects in these mice. With all of these considerations in mind, and given our high success rate with the technique, we highly recommend all future investigations to use the DAT-Cre^{Tg} (or possibly even TH-Cre^{Tg}) midbrain DIO-DREADD models. Heterozygous expression of the transgenic allele allows for relative ease of breeding this manipulation into almost any other genetic background, including *Bmal1*^{-/-} used here, and the virus is readily available, safe, and inexpensive (especially compared to breeding costs of DATKOs). Furthermore the freely available (NIMH Chemical Synthesis and Drug Supply Program, nimh-

repository.rti.org) non-endogenous ligand, Clozapine-N-oxide, readily dissolves in the drinking water at effective concentrations without the need for non-polar vehicle or stabilizing agents. It is of course also injectable or infusible, but requires DMSO at those concentrations necessitating appropriate vehicle controls. The only caution here is that there does appear to be some desensitization to the drug after approximately one week of chronic treatment (see the shortening of active periods in Figure 18) and this is corroborated by other reports in the literature. As we never tested these mice beyond two weeks we could not say how effective it may be longer term but Urban and Roth provide a reasonable argument that it is unlikely to desensitize completely (445). Importantly however, the mice respond immediately to the drug (at least on the relevant timescales being discussed), making it highly suitable to quick investigations for tissue or blood collection at different phases of the clock in order to probe the molecular underpinnings of DUO oscillations. While there is certainly a place for other pharmacological and genetic models, and indeed these can serve as important tools for confirming results, the ease of use of the DREADD model leads us to suggest it as the primary tool for behavioural investigations of DUO dysfunction.