The molecular and neural basis of rest:activity rhythms beyond the circadian range

Pratap Singh Markam

Integrated Program in Neuroscience, McGill University, Montreal

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Abstract

Apart from the well-known 24-hour circadian clock, the sleep-wake cycle in rodents can also be controlled by an alternative oscillatory process. Chronic Methamphetamine (Meth) exposure but also disruption of the dopamine transporter (DAT) gene leads to the emergence of a second rhythmic component which is driven by a circadian clock-independent oscillator. One key characteristic of this oscillator is its tunability: chronic Meth exposure via drinking water at increasing concentrations leads to corresponding rises in locomotor period in rodents. The Meth-dependent rest:activity cycles achieved this way typically fall in the 26-30hrs range however it can also adopt periods reaching into the 100hr range in the absence of the circadian clock. Previous research uncovered that chronic chemogenetic activation of midbrain DA neurons can also lengthen the locomotor rhythm period in the absence of the circadian timer. Based on these findings, we hypothesized that the midbrain DA neurons or a subset of these are necessary for rest:activity rhythm production by the DA oscillator (DO), possibly harboring it.

We found that long-term treatment with Meth at high concentrations can result in infradian rhythms with periodicities ranging from 26-7hrs, corroborating that rhythm production by the DO is highly tunable even in the presence of the circadian timer. Towards identifying the DO substrate, we first genetically ablated the DA neurons of the ventral tegmental area (VTA), a major midbrain DA neuronal subset, and found that ablated mice were incapable of producing infradian rhythms in response to Meth. Loss of infradian rhythm emergence was also observed upon ablation of VTA DA innervations in the nucleus accumbens (NAc). These findings suggest that the VTA-NAc DA circuitry is essential for infradian rhythm emergence. The selective disruption of the gene for tyrosine hydroxylase

(TH), which is essential for DA biosynthesis, in the VTA, also led to the loss of infradian rhythm emergence. On the other hand, elimination of the vesicular monoamine transporter 2 (Vmat2), which is essential for DA vesicular release did not prevent Meth-mediated infradian rhythm emergence.

Together these results indicate that infradian rhythmicity relies on DA production in VTA DA neurons. However, the modus of DA release by these neurons in this context seems unimportant, as neither blockade of DA vesicular release nor DAT disruption prevented infradian rhythm emergence. These data are therefore consistent with the view that DA neurons in the VTA-NAc pathway harbor an oscillator, the DO, which can produce infradian range rhythms in sleep and wake. While these findings advance our knowledge on the biology of infradian rhythms, they also hold importance with regard to the understanding of bipolar disorder (BD). BD patients who exhibit rapid and regular mood cycling show sleep:wake patterns very similar to infradian rhythmic mice. Given that antipsychotic treatment can shorten infradian rhythm period in our mouse model as well as in BD, it seems plausible that the VTA-NAc DA neurons we identified as substrate for infradian rhythm generation in mice also serve as drivers of cyclicity in BD.

Résumé

Outre l'horloge circadienne bien connue avec son cycle de 24h, le cycle veille-sommeil chez les rongeurs peut également être contrôlé par un processus oscillatoire alternatif. Une exposition chronique à la méthamphétamine (Meth), mais aussi la perturbation du gène du transporteur de dopamine (DAT), entraînent l'émergence d'une deuxième composante rythmique pilotée par un oscillateur indépendant de l'horloge circadienne. L'une des principales caractéristiques de cet oscillateur est sa capacité d'adaptation: une exposition chronique à des concentrations croissantes de Meth dans l'eau entraîne une augmentation proportionnelle de la période locomotrice chez le rongeur. Les cycles repos/activité dépendants de la Meth ainsi obtenus se situent généralement entre 26-30h, mais ils peuvent également atteindre des périodes de 100h en l'absence de l'horloge circadienne. Des recherches antérieures ont révélé que l'activation chimiogénétique chronique des neurones dopamine (DA) du mésencéphale peut également allonger la période du rythme de locomotion en l'absence de l'horloge circadienne. Sur la base de ces résultats, nous avons émis l'hypothèse que les neurones DA du mésencéphale ou un sous-ensemble de ces neurones sont nécessaires à la production du rythme repos/activité par l'oscillateur DA (OD), et qu'ils l'hébergent possiblement.

Tout d'abord, nous avons constaté qu'un traitement chronique à long terme avec de fortes concentrations de Meth peut entraîner des rythmes infradiens avec des périodicités allant de 26-72h, ce qui corrobore le fait que la production de rythmes par l'OD est hautement adaptable, même en présence de l'oscillateur circadien. Pour identifier le substrat de l'OD, nous avons d'abord procédé à l'ablation génétique des neurones DA de l'aire tegmentaire ventrale (ATV), un sous-ensemble majeur de neurones DA du

mésencéphale, et avons constaté que les souris lésées étaient incapables de produire des rythmes infradiens en réponse à la Meth. La perte de l'émergence du rythme infradien a également été observée lors de l'ablation de l'innervations DAergique de l'ATV vers le noyau accumbens (NAc). Ces résultats suggèrent que le circuit DA ATV-NAc est essentiel pour l'émergence du rythme infradien. La perturbation sélective dans l'ATV du gène de la tyrosine hydroxylase (TH), enzyme essentielle à la biosynthèse de la DA, a aussi entraîné la perte de l'émergence du rythme infradien. En revanche, l'élimination du transporteur vésiculaire de monoamines 2 (Vmat2), qui est essentiel pour la libération vésiculaire de DA, n'a pas empêché l'émergence du rythme infradien induite par la Meth.

L'ensemble de ces résultats indique que la rythmicité infradienne repose sur la production de DA dans les neurones DA de l'ATV. Cependant, le mode de libération de DA par ces neurones ne semble pas important dans ce contexte, car ni le blocage de la libération vésiculaire de DA ni la perturbation du DAT n'ont empêché l'émergence du rythme infradien. Ces données sont donc compatibles avec l'idée que les neurones DA de la voie ATV-NAc abritent un oscillateur, l'OD, qui peut produire des rythmes infradiens dans le sommeil et l'éveil. Si ces résultats font progresser nos connaissances sur la biologie des rythmes infradiens, ils sont également importants pour la compréhension des troubles bipolaires (TB). Certains patients atteints de ces troubles présentent des cycles d'humeur rapides et réguliers, avec des patrons de veille et de sommeil très similaires au rythme infradien chez les souris. Étant donné que le traitement antipsychotique peut raccourcir la période du rythme infradien dans notre modèle de souris ainsi que dans le TB, il semble plausible que les neurones DA ATV-NAc que nous avons identifiés comme substrat pour

la génération du rythme infradien chez les souris sont également des moteurs de la cyclicité dans le TB.

Dedicated to my late grandfather, ALAL and grandmother, INDIRA

- Dada, Dadi, Aapki yaadein humesha taaza rahengi

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Contribution to the original knowledge

This work presents the first evidence for the neural basis of a dopamine-based oscillator (DO) that drives arousal, also referred to as Methamphetamine-sensitive circadian oscillator (MASCO). We demonstrate that the ablation of the VTA-NAc dopaminergic circuitry abolishes the capacity to generate a 2nd rhythmic locomotor component exhibiting infradian range periodicities. We also demonstrate that tyrosine hydroxylase, an enzyme critical for dopamine biosynthesis, in the VTA is necessary for induction of the 2nd rhythmic component while the vesicular monoamine transporter 2, an enzyme critical for vesicular packaging and synaptic release of dopamine, is not necessary. To summarize, this thesis provides evidence for the neural basis and a key molecular component of a second oscillator that drives rest:activity rhythms independent of the circadian clock. As the rest:activity patterns generated by this dopamine-based oscillator show similarities to sleep:wake aberration found among bipolar disorder patients, our work may provide new avenues towards a mechanistic understanding of sleep perturbations observed in bipolar disorder and related psychopathologies.

This thesis led to a manuscript under submission:

> **Markam, P. S**.*, Bourguignon, C.*, Zhu, L., Darvas, M., Kokoeva, M. V., Giros, B., Sabatini, P., & Storch, K.-F. (to be submitted). The neurons that drive sleep-wake rhythms beyond the circadian range.

Contribution of Authors

Dr. Kai-Florian Storch and I conceptualized the work presented in chapter 3,4,5 and 6. I designed the experiments with Dr. Storch's support and under his supervision, I performed all the experiments for chapter 3, 4, and 5. For chapter 6, Dr. Lei Zhu and I both contributed equally towards conducting the experiments. I performed the analysis of all experiments presented with assistance from Dr. Clément Bourguignon and Dr. Kai-Florian Storch.

Dr. Lei Zhu provided assistance with animal maintenance, animal perfusion, brain collection and pharmacological manipulations. Dr. Zhu performed genotyping for all the animals and both Dr. Zhu and I contributed to colony maintenance.

This thesis also includes portions of manuscript titled "The neurons that drive sleep-wake rhythms beyond the circadian range" (Markam and Bourguignon et al, to be submitted); Pratap Singh Markam, Clément Bourguignon and Kai-Florian Storch wrote the initial draft of the manuscript; Pratap Singh Markam, Clément Bourguignon, Lei Zhu, Kai-Florian, Bruno Giros, Maia Kokoeva, Paul Sabatini and Thomas Darvas contributed to editing and revising the manuscript. In this work, Pratap Singh Markam, Clément Bourguignon and Kai-Florian Storch contributed to experiment designs; Pratap Singh Markam and Clément Bourguignon performed all surgeries, histology, behavior recordings and data analysis; Lei Zhu provided technical assistance and performed genotyping of all the transgenic lines; Kai-Florian Storch supervised the experiments. All authors contributed to data interpretation.

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List of Abbreviations

2ndC	2nd rhythmic component
AAV	Adeno-associated virus
BD	Bipolar disorder
DA	Dopamine
DD	Constant darkness
DO	Dopaminergic oscillator
DUO	Dopaminergic ultradian oscillator
LA	Locomotor activity
LC	Locus coeruleus
LD	Light-dark cycle
LS	Lomb-Scargle
MASCO	Methamphetamine sensitive circadian oscillator
Meth	Methamphetamine
NAc	Nucleus Accumbens
SCN	Suprachiasmatic Nucleus
SN	Substantia Nigra
TH	Tyrosine Hydroxylase
TTFL	Transcriptional translational feedback loop
Vmat2	Vesicular monoamine transporter 2
VTA	Ventral Tegmental Area

Introduction

Foundation:

Physiological activities in many species are oftentimes rhythmic in nature as a reflection of adaptation to the light-dark cycle on earth. These biological rhythms follow the 24hr period of the solar day and still persist in the absence of timing cues but with a period slightly shorter or longer than 24hr, hence termed as circadian (from the Latin circa diem = about a day). Interestingly, there are biological rhythms in the ultradian (shorter than a day) or infradian (longer than a day) range as well. For instance, the rest:activity patterns in voles and premature infants exhibit a strong ultradian rhythmicity with periods in the 2-4hr range (Gerkema & van der Leest, 1991; Rivkees et al., 2004), while infradian rhythms in rest-activity cycle have been reported in psychiatric illnesses such as bipolar disorder (Wehr et al., 1982; Welsh et al., 1986). While the key neural substrate for the generation of circadian rhythmicity in mammals, the suprachiasmatic nucleus (SCN), has been identified decades ago (Stephan & I. Zucker, 1972), the brain areas or cell populations, which drive these non-circadian ultradian and infradian rhythms are still unknown.

Infradian rhythms in sleep-wake or rest-activity can also manifest as a 2^{nd} rhythmic locomotor component (2ndC) besides the circadian component when mice are chronically supplemented with a psychostimulant such as Methamphetamine (Meth) in the drinking water. This convenient model has long since been used for studying the physiological basis of these Meth-associated locomotor rhythms. Early work on Meth-dependent rhythms revealed that they must be driven by an oscillator which has an SCN-independent origin. The most widely used term for this oscillator to this date is

Methamphetamine sensitive circadian oscillator (*MASCO*), which is in part based on the observation that the locomotor rhythms the oscillator generates typically exhibit circadian range rhythmicity in SCN-lesioned mice when Meth is supplemented (K. I. Honma et al., 1987; Tataroglu et al., 2006). More recent, work revealed a critical involvement of the dopamine system in the generation of these Meth-induced SCN-independent rest-activity rhythms (Blum et al., 2014). The underlying oscillator was referred to as the dopaminergic ultradian oscillator (DUO) as the rest:activity rhythms it drives were found to be ultradian in clock deficient mice (Blum et al., 2014). Work on the DUO showed that the DUO is highly tunable able to adopt periodicities, from ultradian to circadian and infradian range. The shift to longer periods was found to correlate to the Meth concentration supplemented in the drinking water. This work suggested that that the circadian feature in MASCO rhythms in circadian clock-deficient mice represents in fact a long-period (circadian range) manifestation of the DUO. This work further showed that an infradian 2ndC could also emerge in the absence of Meth, upon disruption of the dopamine transporter (DAT) gene (Blum et al., 2014).

Revision of terminology:

Throughout the thesis, we will use the term ‘dopaminergic oscillator‘(DO) rather than DUO when describing the oscillatory process underlying the 2ndC that results from Meth treatment or DAT disruption. We believe that DUO is a too narrow descriptor as it is not clear if ultradian rhythm generation is indeed the default mode of operation for this oscillator.

Rationale:

A 2ndC is typically not observed in laboratory rodents under standard condition, however DAT disruption or chronic Meth treatment via drinking water can lead to locomotor period lengthening beyond 24hr and ultimately to the emergence of a 2ndC. Notably, the 2ndC that emerges upon Meth treatment has been reported to typically adopt periods around ~26-30hrs with occasional account of periods up to 48hrs (Taufique et al., 2022). Thus, the previous literature did not provide a unifying view on the actual period range the 2ndC can adopt, leaving the tunability aspect of the underlying oscillator obscure. Also, while previous work clearly implicated the dopamine system in the emergence of 2ndCs (Blum et al., 2014), the specific structures and cell populations that are necessary for the generation of 2ndC remained ambiguous. Moreover, since both, blockade (DAT KO) and reversal (induced by Meth) of dopamine reuptake into DA neurons could induce a 2ndC (Blum et al., 2014), it seems as if there is no specific requirement with regard to the mode of DA release, as long as high levels of extracellular DA can be attained, which seems to be the likely initiating event for DO activation and 2ndC emergence.

Objective:

The objective of my thesis is to further characterize 2ndC generation and DO function. I aim to examine the tunability aspect of the DO that is its ability to acquire wide range of periodicities, specifically in mice with an intact circadian timer. Furthermore, I wish to determine the neuronal substrate of 2ndC generation and the DO. To this end, we employ genetic mouse models and viral approaches to manipulate selected neuronal populations in the brain within the dopamine system. To assess their ability for 2ndC induction, mouse locomotor behavior will be monitored long-term by recording running wheel activity in

conjunction with pharmacological treatments such as Methamphetamine exposure in the drinking water.

The aims of my thesis are as follows:

1) To demonstrate that the Meth-induced 2ndC shows an extremely wide period range as opposed to the circadian timer

Hypothesis: The rhythmicity of the Meth-induced 2ndC is not limited to the circadian range even in the presence of a circadian timer but instead shows a wide range of periodicities which can reach far into the infradian range.

2) To identify the neurons that are necessary for driving the Meth-induced 2ndC

Hypothesis: DA neurons of the ventral tegmental area (VTA) and their ability to synthesize dopamine are necessary for 2ndC emergence

3) To identify the projection site of the VTA DA neurons that are necessary for driving the 2ndC

Hypothesis: DA neuron that project to ventral striatum, i.e., the nucleus accumbens region, are necessary for 2ndC emergence.

4) To assess the modalities of DA release in the context of 2nC induction

Hypothesis: Vesicular DA release is not required for the generation of the 2ndC.

Chapter 1: Literature Review

1.1 Biological rhythms of rest and activity

Biological events that recur at regular intervals are considered biological rhythms. They can occur at a scale of milliseconds to several hours and years. Some rhythms are limited to the molecular and cellular level, such as electrical activity in neurons or the cell cycle, while others can extend into behavior as in case of hibernation or bird migration (Goldbeter, 2008).

Life on earth has evolved under the influences of i) the daily 24hr light-dark changes due to earth's rotation, ii) the annual latitude dependent seasonal changes in the duration of the daily light period due to the earth rotation around the sun at an oblique angle, iii) the 12.4 hrs cycle in tidal ebb and flow due to the changes in moon-earth gravitational pull (Wilcockson & Zhang, 2008) and, iv) the 29.5 day lunar illumination and gravitational cycle (Raible et al., 2017). The various rhythmic behaviors observed throughout the living world can thus be seen as evolutionary adaptations to these geophysical periodicities either to optimize coping with the ensuing changes in the environment or using the geophysical periodicities as timing signals to promote important events such as mating (Andreatta & Tessmar-Raible, 2020)

Rest and activity rhythms are one such widely observed biological phenomena and its relative coordination with geophysical cycles dictate physiology and other behaviours in several species. There is a daily or *diurnal cycle* of rest and activity observable in most species, which interestingly falls in 2 large categories with respect to the phase positioning of the cycle. Laboratory rodents as nocturnal species are active during the

daily dark period whereas diurnal human are active during the day (Foster et al., 2020). A prime example for *tide-aligned rhythms* is provide by the Fiddler crabs, which emerge along the shores during low tides to forage and mate while green shore crabs wait for their foraging activity during high tides (Wilcockson and Zhang, 2008). Association with *lunar rhythms* is demonstrated by the mating behaviour of non-biting midges of *Clunio* genus which surface from the sea en masse every full moon for their nuptial dance (Kaiser et al., 2016) while the twice yearly migration of birds such as the *Sylvia* warbler, represents a classic example of a seasonal rhythm (Gwinner, 1996). Importantly, these biological rhythms are often found to persist under constant conditions, i.e., under conditions where the timing cue is removed. Humans and laboratory rodents would still exhibit daily rhythms in rest-activity even when exposed to constant darkness across days (Aschoff, 1965; Pittendrigh & Daan, 1974). Likewise shore crabs, midges and garden warblers would still show their respective tidal, lunar, and seasonal rhythms in physiology in the absence of geophysical-driven tidal, lunar illumination, and photoperiodic changes (Gwinner, 1996; Kaiser et al., 2016; Wilcockson & Zhang, 2008). Under such constant conditions, the period (τ) displayed typically slightly deviates from the period of the respective geophysical cycles they are normally entrained to. This has led to the use of the prefix circa (about) in their terminologies: circadian (about a day) (Halberg, 1969), circatidal, circalunal, and circannual rhythms, respectively.

Rhythm in the hours to multi-day range, have been sub-categorized as ultradian, circadian, and infradian rhythms, respectively, with ultradian rhythms encompassing the 1 hour to 20hr range, followed by circadian rhythms defined by a period range from 20-

28hrs, and infradian rhythms with periods greater than 29hrs (Aschoff, 1978; Halberg, 1969).

Table 1 Rhythms of sleep and activity

Ultradian range (<20h)	Circadian range (20-28h)	Infradian range (>28h)
Vole activity and feeding patterns (Gerkema & van der Leest, 1991)	Leaf movement (Jj, 1729)	Estrus cycle (Alvord et al., 2022)
Ultradian locomotor components in mouse (Dowse et al., 2010)	Locomotor activity in many species, including bacteria, fungi, flies , and mammals (Panda, Hogenesch, et al., 2002)	Bird migration (Gwinner, 1996)
Human infants (Rivkees, 2003)		Hibernation (Geiser, 2004)

Early evidences for a circadian activity rhythm resulted from the study of *Mimosa Pudica*, whose daily rhythms in vertical leaf motion was found to persist in constant darkness (Jj, 1729). Since then, monitoring movements, or more specifically locomotor activity has been established as a robust and easily accessible read out of the circadian clock activity specifically across the animal kingdom.

In humans, wrist-actigraphy has become a widely accepted means to explore the diurnal and circadian elements of daily activity. Simultaneous recording of EEG validated wrist-actigraphy as a relatively accurate means to determine sleep parameters such as sleep

on- and off-sets as well as sleep lengths and phasing (Lavin-Gonzalez et al., 2020; van Hees et al., 2018). Importantly, sleep can be recorded this way over multiple days to months objectively. Its automated nature enables behavioural recording without the influence of the observer and the study subject (Ancoli-Israel et al., 2003). In rodents, locomotor activity is recorded by a variety of modes such as infrared-beam breaking, running wheel engagement or by telemetry implants. To enable visual inspection of rhythmic patterns derived from recordings over weeks to month, the activity is plotted minute by minute in the form of an actogram with the y-axis representing 24hrs and the x-axis, successive days. For better visualization of patterns, a double-plot format is frequently chosen, i.e. the x-axis represents 2 successive days, where the second day is replotted in the next line (Fig. 1.1, adopted from Jud et al., 2005). Double or even triple or quadruple plots can be extremely useful for visual examination of rhythmic activity

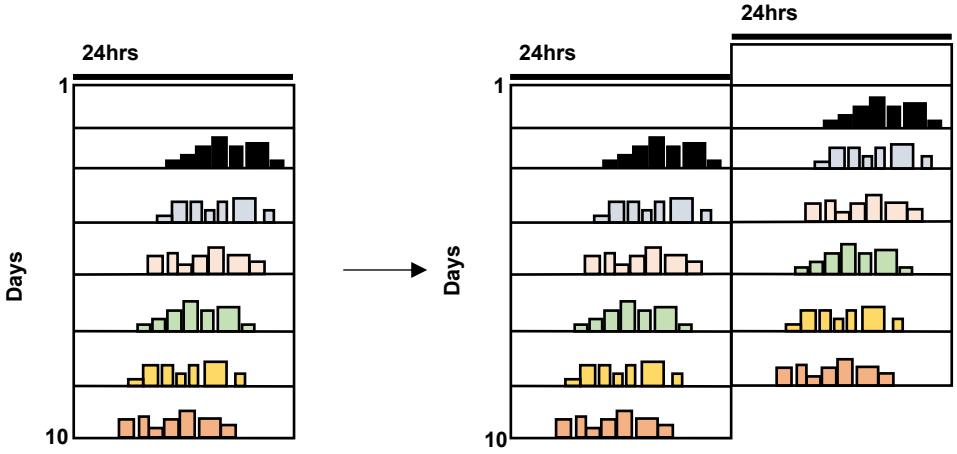


Figure 1.1 : Double plotted actogram display. Adapted from Jud et al., 2005

Shown here on the left are are the daily running wheel counts represented by rectangular blocks. When two successive days are plotted along one row, it is referred to as a double plotted actogram as displayed on the right.

patterns over time providing information on one or multiple rhythmic components and their onset or phases with respect to a zeitgeber (an entrainment cue) if present.

1.2 Biological clocks

Biological rhythms are aligned to pre-existing geophysical rhythms driving animals towards favourable times for wake and sleep, respectively. However, while typically aligned to geophysical rhythms, biological rhythms still persist in the absence of geophysical cues. The observation of sustained rhythmicity in the absence of environmental change argues for an endogenous origin of these rhythms in rest:activity. The early observation of circadian leaf movements already indicated that an endogenous circadian oscillator or clock must exists which drives ~24hr rhythms. Indeed, almost a century later, first anatomical evidence of an endogenous circadian clock was provided by hypothalamic lesion studies in rats (Moore & Eichler, 1972; Stephan & I. Zucker, 1972). Hypothalamic lesions were only found to yield a loss of circadian rhythms in locomotor and drinking behavior, as well as circulating cortisol levels, when the suprachiasmatic nucleus (SCN) structure was included in the lesioned area. These studies set the foundation for the SCN as the site of the central circadian pacemaker in mammals which acts as the chief controller of the daily rhythm in sleep and wake (Dibner et al., 2010).

1.2.1 Circadian clock

The rhythms in physiology and behavior that exhibit ~24 hrs periodicities are referred to as circadian rhythms and have likely evolved in consequence to the day-night variation caused by the earth's rotation (Aschoff, 1965). The autonomous circadian rhythm generator which is chiefly responsible for the production of ~24hr sleep:wake rhythms is located in a paired structure at the base of the anterior hypothalamus just above the optic chiasm, called the SCN (Ibuka & Kawamura, 1975; Moore, 1973; Moore & Eichler, 1972; Stephan & I. Zucker, 1972).

Specific environmental cues, also termed zeitgebers, align the biological clock with geophysical rhythms. This type of alignment is also known as entrainment of the clock (Pittendrigh, 1960). Light is the predominant zeitgeber for circadian clock entrainment to the solar 24hr day. The exact entrainment to the 24hr solar cycle can be accomplished over wide range of light intensities, thereby enabling alignment with the solar day throughout the seasons and across most latitudes. The solar-day entrainment of the circadian clock ensures that the organism's rhythmic physiology is optimally phase positioned to maximize adaptation to the profound daily environmental changes. Notably, even a single light pulse administered at the same time each day is sufficient to entrain the circadian activity rhythms of a laboratory rodent (Pittendrigh, 1960). However, in the absence of any 24hr zeitgebers, the animal still exhibits a daily rhythm of sleep and wake however its period is solely dictated by the period of the endogenous circadian clock which is ~24hrs. For example, the human circadian oscillator cycles at periods that are slightly longer (~24.5hr) than the solar day, whereas laboratory rodents typically exhibit endogenous periods that are shorter (~23.5hr) than a day (Aschoff, 1965; Pittendrigh, 1960; Pittendrigh & Daan, 1974).

The circadian clock is cell autonomous, e.g., circadian rhythms in electrical activity are still observable in individual SCN neurons upon dissociation when recorded from in a culture dish (Welsh et al., 1995). The circadian rhythmicity is a product of a transcriptional translational feedback loop (TTFL) within the SCN neurons but is also present in SCN astrocytes and other cell-types in tissues throughout the body (Brancaccio et al., 2019; Mohawk et al., 2012; Rusak & Zucker, 1979; Welsh et al., 1995; Yoo et al., 2004). The autoregulatory TTFL (Takahashi, 2017) consists of circadian clock genes that also

engage the rhythmic expression of output genes whose transcripts mediate circadian information into the proteome and ultimately to other brain areas and throughout the body axis (Miller et al., 2007; Panda, Antoch, et al., 2002; Reppert & Weaver, 2002; Storch et al., 2002; Takahashi, 2017).

The core clock genes include Bmal1, CLOCK, Pers and Crys that are essential for driving the TTFL and thus circadian rhythmicity (Ko & Takahashi, 2006). At its core, circadian rhythm generation in clock cells relies on the transcriptional activators Bmal1 and Clock which heterodimerize and selectively bind to the E-box promoter element to initiate the transcription of other core clock components, specifically the *Per* genes, *Period* 1 and 2 and the *Cry* genes, *Cryptochrome* 1 and 2 (Bunger et al., 2000; Gekakis et al., 1998; Hogenesch et al., 1998; King et al., 1997; Kume et al., 1999; Zheng et al., 2001) which when translated also heterodimerize as well. The heterodimer of Pers and Crys then negatively regulate their own transcription by facilitating the release of Bmal1:Clock complex from the E-box domain. This transcriptional inhibition leads to gradual reduction in Per and Cry protein production which then results in the net loss of these proteins due to continuous degradation. This eventually leads to the disinhibition of Bmal1:Clock transcriptional activity starting Pers and Crys production anew (Griffin et al., 1999; Kume et al., 1999; Shearman et al., 2000; van der Horst et al., 1999; Vitaterna et al., 1999). This TTFL process goes through one cycle every ~24hrs resulting in circadian rhythmicity (Takahashi, 2017).

While *Bmal1* deletion is sufficient to fully abolish TTFL functionality (Hogenesch et al., 1998), with regard to the *Crys* and *Pers*, the deletion of all paralogs (except *Per*3) of either one are required to disrupt the circadian oscillator. Animals with such genetic

manipulations completely lose circadian control of sleep and wakefulness and are thus considered arrhythmic (Bae et al., 2001; Bunger et al., 2000; Van Der Horst et al., 1999). Of note, the resulting behavioral pattern in Bmal1KO mice was found to often still exhibit rhythmic elements in the ultradian range albeit with greatly varying periodicities centering in 2-5hrs range (Blum et al., 2014).

1.2.2 Dopaminergic (ultradian) oscillator (DO)

1.2.2.1 Ultradian rhythms in physiology

Evidence for ultradian regulation in bodily processes has been reported across various vertebrate and non-vertebrate species, encompassing sleep-wake, hormonal release, core body temperature, and foraging activities among others (Daan & Slopsema, 1978; Dudley et al., 2003; Eastman et al., 1984; Stavreva et al., 2009; Tannenbaum & Martin, 1976). When circadian and ultradian rhythms are studied in concert, they often appear to occur in harmony, i.e., they seem stably locked at a certain phase relationship. There is clear evidence for this in rest:activity patterns of infants (Rivkees et al., 2004) or the common vole (Daan & Slopsema, 1978; Gerkema & van der Leest, 1991). The robust ultradian activity of the common vole is thought to allow for synchronized and rhythmic foraging every 2.7 hours during daytime among voles which in turn reduces predation risk (Daan & Slopsema, 1978). Population synchrony during foraging as so prominently exhibited by the vole might represent one general driving force behind the evolution of ultradian rhythms (Daan & Slopsema, 1978). This might be specifically beneficial for gregarious species as demonstrated by the Svalbard reindeer during periods of constant light or darkness (van Oort et al., 2007).

The period stability and expression robustness of ultradian activities in the common vole suggests that these rhythms are the output of an oscillator instead of merely being the product of an hourglass process (Gerkema & van der Leest, 1991). Consistent with this view, the ultradian period of food hopper approaches is not affected when the actual food intake is blocked in the vole, indicating that this activity is not simply triggered by e.g. energy status-based periodic hunger (Geiser, 2004).

1.2.2.2 Basis for the DO

Methamphetamine (Meth) is a psychostimulant drug that targets the catecholamine system primarily monoamine transporters, reversing the uptake of the monoamines DA and NE thereby increasing their extracellular levels (Seiden et al., 1993; Sulzer et al., 2005a). Along with its association to addiction (Sulzer et al., 2005a), Meth is also known to disrupt circadian rhythmicity in rodents and thus affecting the sleep-wake cycle of the animal. First shown in rats, supplementing drinking water with Meth resulted in the dampening of circadian rhythms and the concurrent emergence of a second consolidated rhythmic locomotor component in the circadian or near-infradian range (K.-I. Honma et al., 1986). This Meth-induced activity rhythm persisted even in animals where central pacemaker function was disrupted by SCN lesion or genetic impairment (K. I. Honma et al., 1987; Mohawk et al., 2009). Since this rhythmic activity was only detectable during Meth treatment, it was first dubbed the Methamphetamine-induced oscillator. However, it was alternatively speculated to be the result of rhythmic drinking by animals, i.e., driven by the drinking behavior which may or may not be under rhythmic control. It was further suggested that Meth may produce arousal and that the rhythm is produced by the subsequent exhaustion which resulted in extended sleep upon which the animal will

resume wakefulness and thus drinking. This way the Meth-dependent rhythm in locomotion could be the sheer result of cyclical exhaustion thus arguing against an endogenous oscillator as a source. However, the drinking cycle as a driver was ruled out when infradian rhythmicity could be induced even when Meth was continuously supplied via a micropump (K.-I. Honma et al., 1987). As the period of the second rhythmic component was found to be greater than 24hrs but typically not by a great margin, regardless if the circadian timer was present or not, its descriptor was then refined to *Methamphetamine-sensitive circadian oscillator*, or MASCO (Tataroglu et al., 2006).

As Meth in addition to targeting NE neurons also acts on the dopamine system by blocking the dopamine transporter (Fumagalli et al., 1998; Giros et al., 1996a), it seemed conceivable that DA neurons were involved in the MASCO rhythm. Dopamine as well as the other monoamines, histamine, serotonin, and norepinephrine which are all synthesized in discrete nuclei/areas of the brain stem/diencephalon, have been considered to be a part of the ascending arousal pathway (Saper et al., 2005). Notably, pharmacological or genetic manipulations affecting DA levels in the CNS can lead to severe locomotor abnormalities while altering brain levels of the other monoamines only leads to comparably mild locomotor phenotypes (Bengel et al., 1998; Isingrini et al., 2016; Parmentier et al., 2002; Thomas & Palmiter, 1997; Xu et al., 2000). Given the literature support of the importance of dopamine over the other monoamines in the context of arousal/locomotor activity regulation in conjunction with the fact that MASCO-like activity strictly depends on chronic Methamphetamine treatment it seemed plausible that the MASCO activity generation involves DA neurons.

1.2.2.3 From MAO/MASCO to DUO/DO

Circadian activity rhythms show tremendous period stability and robust expression under constant conditions (constant darkness) while the ultradian locomotor rhythms appear much more labile in mice and rats. Perhaps due to this lability, ultradian locomotor rhythms had received little attention by the research community in the past and ultradian patterns were therefore often simply considered arrhythmic patterns (Bunger et al., 2000). While ultradian locomotor activity superimposed on circadian locomotor activity can be discerned in laboratory animals, eliminating the circadian timer and the environmental light dark cycle enables an 'unobstructed' view on ultradian locomotor rhythms, facilitating their study (Abrahamson & Moore, 2006; Bunger et al., 2000; Eastman et al., 1984; Ibuka & Kawamura, 1975; Van Der Horst et al., 1999). Through a series of experiments aimed at monitoring and manipulating dopamine tone/dopaminergic neurons of the midbrain, our lab showed that the sleep:wake pattern is ultradian in nature more often than not in the absence of the circadian clock and that these ultradian locomotor cycles are associated with synchronous fluctuations in extracellular dopamine levels in the striatum. The data further supported the notion that the underlying oscillatory process driving ultradian rhythms is highly tunable, i.e., the oscillator seem to be capable of producing rhythms in locomotor activity at a wide range of periods ranging from a few to 100hrs. This tunability was demonstrated by chronic Methamphetamine treatment of circadian clock deficient Bmal1KO mice via drinking water in constant darkness. The animals exhibited gradual lengthening of ultradian locomotor rhythms as the dosage of Meth in water was incrementally increased, ranging from 12 hrs to 48 hrs and eventually reaching periodicities beyond 100hrs (Blum et al., 2014). This observation provided support for

the notion that the MASCO rhythms described for the first-time a few decades ago are in fact long-period manifestations of an ultradian rhythm generator. The lack of acknowledgement for the evidently wide tuning range of this oscillator in the preceding literature can be plausibly attributed to the consistent usage of relatively low Meth concentrations in the drinking water which prevented the oscillator from adopting long infradian rhythmicities and instead resulted in periodicities which seem to be limited to the circadian range. This may have led to the addition of the 'circadian' attribute to the MASCO term (Methamphetamine-sensitive circadian oscillator) (Tataroglu et al., 2006; Taufique et al., 2022).

The putative link between MASCO rhythms and dopamine was critically confirmed by the observation of a 2ndC upon disruption of the dopamine transporter (DAT) gene that quite resembled 2ndC resulting from Meth treatment. Moreover it was found that in the absence of the circadian clock, such DAT disruption led to the lengthening of ultradian locomotor periods from ~4-6hrs to ~12-14hrs (Blum et al., 2014). Chemogenetic activation of DAT-positive, midbrain dopamine neurons also led to a lengthening of the ultradian period similar to DATKO. While manipulations that elevated extracellular DA tone and DA postsynaptic signalling appeared to be associated with longer ultradian periods, lowering postsynaptic DA action by antipsychotic treatment led to period shortening. When the drinking water was supplemented with haloperidol, an antipsychotic drug along with Meth, Meth-induced period lengthening was counteracted. Together, the lab's findings pointed to the existence of an oscillator secondary to the circadian clock whose operation and output transmission likely relies on dopamine, and which is characterized by a high degree of period tunability. Furthermore, the fact that DAT gene disruption as well as

chemogenetic activation in midbrain DAT neurons lengthened the ultradian locomotor period suggested that the oscillator is located within the DAT neurons of the midbrain (Blum et al., 2014).

While ultradian rhythms are overtly detectable in the absence of a functional circadian clock under constant dark conditions, Blum et al. (2014) also found evidence that the ultradian oscillator is operative in the intact mouse when exposed to a light:dark cycle. Circadian clock intact mice more often than not exhibit a distinct temporal distribution of activity during their wake phase with 3 relatively equidistant activity peaks spread out across the night phase and DAT disruption led to an abrogation of these three-peak nighttime activity pattern. Together, it was concluded that the ultradian oscillator cycles in synchrony with the circadian clock and that this harmony may be perturbed when the DUO (dopaminergic ultradian oscillator) cycles at longer, non-harmonic periods, whereas a harmonious relationship with the 24hr circadian timer can be again established when the DUO reaches periodicities of 24 or 48 hrs (Blum et al., 2014).

1.2.2.4 DO driven infradian rhythms in the presence of the circadian clock

As the DA-dependent oscillator operates independent of the circadian timer and is able to adopt periods far into the infradian range, we felt it would be more appropriate to re-label the oscillator as the dopaminergic oscillator or DO. Notably, the infradian manifestations of the DO is observed when DAT is disrupted or during chronic Meth treatment (Blum et al., 2014) both of which target the DA system resulting in increased DA concentration in the extracellular space and reduced intracellular DA. Interestingly, the period lengthening of the DO by increasing Meth concentrations (Taufique et al., 2022) seems somewhat attenuated in mice with an intact circadian timer as opposed to

clock deficient mice (Blum et al., 2014). The most frequently used Meth concentration is 0.005% (50mg/L) resulting in a 2ndC with period ranging from 26-30hrs with occasional 48-55hr period when the Meth concentration in the drinking water is doubled to 0.01% (100mg/L). However, in the absence of the circadian timer, e.g., by disruption of the Bmal1 gene or SCN lesion, mice seem to more readily adopt periods of 48 and beyond at 100 mg/L, with individual animals reaching periods of 100 hrs. This seemingly wider tuning range or facilitation of DO period lengthening in clock-deficient mice could plausibly be due to the lack of crosstalk between the circadian clock and the DO. That the SCN and the DO can affect each other's phasing is demonstrated by the observation of relative coordination of the 2ndC. In the presence of the circadian clock the 2ndC shows period lengthening whenever the 2ndC (and thus the DO) and the circadian clock come into temporal phase alignment. Once the 2ndC and circadian clock phase are distant enough from one another, the 2ndC period shortens again. Such pattern can only be plausibly explained by assuming that the circadian clock is able to partially entrain the DO, which results in the above-described pattern of relative coordination (Blum et al., 2014; Bourguignon & Storch, 2017). Thus, the circadian clock is typically 'slowing' down the DO intermittently (when the two oscillators temporally phase align) which can explain why at the identical Meth concentration in the drinking water, the DO shows an overall longer period in mice lacking a circadian timer versus intact mice (Blum et al., 2014). Interestingly, there is also evidence for the reciprocal effect, i.e., the DO is able to partially entrain the circadian timer. When mice are exposed to Meth under constant darkness then the phase of the circadian timer is typically shifted to longer hours after extended

time of Meth exposure, i.e., the DO temporally lengthens the circadian clock period (Miyazaki et al., 2021)

1.3 Dopaminergic system and DO

1.3.1. Dopamine system

Dopamine along with epinephrine (adrenaline) and norepinephrine (NE) (noradrenaline) are classified as catecholamines due to their shared structural similarities, a catechol-side-chain amine. The catecholamines share common catecholamine synthesizing enzymes, tyrosine hydroxylase (TH), a rate limiting enzyme for dopamine synthesis and dopamine decarboxylase (DDC) (Goldstein, 2010).

The biosynthesis pathway of dopamine involves tyrosine as the initial substrate which is converted to L-dihydroxyphenylalanine (L-DOPA) by TH and then decarboxylated by DDC to form DA. NE neurons of the locus coeruleus, further convert the produced DA to NE by the dopamine-beta-hydroxylase (DBH) enzyme (Goldstein, 2010).

In the DA neurons, the synthesized DA is packaged into vesicles via the vesicular monoamine transporter 2 (Vmat2) to facilitate its synaptic release. After postsynaptic receptor activation, DA is taken up from the synaptic cleft into the presynaptic DA neuron by action of the dopamine transporters for its recycling or degradation (Eiden et al., 2004).

1.3.2 The role of VTA DA neurons in arousal

The major dopamine neuron populations that are involved in regulation of sleep and wakefulness are the ventral tegmental area (VTA), the substantia nigra pars compacta (SN) and the dorsal raphe DA neurons. All the three neuronal populations exhibit increased burst firing during wake state but DA neurons of the VTA are also active during

REM sleep (Brown et al., 2012; Cho et al., 2017). Psychostimulants that enhance dopaminergic tone, i.e., increase extracellular DA levels, also promote prolonged wakefulness (Wisor et al., 2001) whereas suppressing the neuronal excitability of VTA DA neurons can attenuate both wakefulness and REM sleep, promoting NREM sleep instead (Eban-Rothschild et al., 2016a). The inhibition of VTA DA neurons also promotes nest-building behavior suggesting that it is not just the absence of VTA DA-mediated locomotor drive that results in increased sleep, but that this inhibition unleashes in addition a behavioral program that is negatively regulated by these VTA DA neurons. Consistently, optogenetic stimulation of VTA DA neurons initiates and maintains wakefulness while inhibiting nest building during the stimulation period. Whereas VTA DA neurons projects to various brain areas, only their NAc projections seem capable of maintaining VTA stimulated arousal suggesting that the VTA-NAc circuitry plays a critical role in sleep-wake architecture regulation (Eban-Rothschild et al., 2016a). Notably, the VTA-NAc circuitry communicates with another major arousal system, the orexin neurons in the lateral hypothalamus thereby forming a feedback loop to facilitate wakefulness maintenance (Sulaman et al., 2023). Aside from promoting wakefulness, chronic activation of midbrain DA neurons can also lengthen rest-activity rhythms in clock-deficient mice displaying ultradian rhythmicity, consistent with the presence of an arousal-inducing oscillator in the midbrain (Blum et al., 2014). This rhythm generator as discussed above likely represents a highly tunable dopaminergic oscillator and given the evidence for the role of VTA DA neurons in maintaining arousal and inducing arousal rhythm changes, this neuronal population is the most probable candidate for the neural substrate of DO.

1.4 DO infradian rhythms and psychiatric disorder

The interaction between the circadian clock and the sleep homeostat shapes the pattern of sleep and wakefulness in most species including humans. Under conditions with predictable recurrence of the daily environmental changes such as the solar light:dark cycle, these two systems together ensure that there is little day-to-day variation in sleep length and phasing. However, this 'homeostatic' state, can be disrupted by extrinsic as well as intrinsic factors that either targets the interaction or affects the two physiological process individually thereby causing sleep disturbances (Deboer, 2018).

In humans, sleep disturbances can be manifested in the form of irregular sleep and wake onsets, frequent awakening at night or daytime insomnia (Partinen, 2011). Sleep aberrations can also affect the periodicity of the sleep-wake cycle resulting in sleep rhythms that deviate from 24hrs (Malkani et al., 2018; Quera Salva et al., 2017). One such aberration is known as non-24hr sleep-wake disorder (N24SWD) and is characterized by a sleep-wake rhythm with a period significantly longer than 24hrs often reaching far into the infradian range (Emens et al., 2022). Furthermore, among blind subjects there are those who are considered totally blind. In addition to their image forming blindness these subjects also suffer from circadian blindness (Lockley et al., 2007) likely due to the loss of function of the intrinsically photosensitive retinal ganglion cells (Berson et al., 2002), which are known to represent the principal conduits of non-image forming vision (Ecker et al., 2010). Such totally blind subjects show sleep-wake cycles with a period of slightly longer than 24hrs suggestive that their circadian clock free run, i.e., their sleep wake rhythm now solely follows the periodicity of the intrinsic circadian

clock which is no longer entrained to the light dark cycle (Lockley et al., 2007). The mechanistic basis and cellular origins for N24SWD on the other hand are still enigmatic.

Interestingly, evidence for infradian rhythmicity in sleep-wake has been also reported in the context of bipolar disorders (Wehr et al., 1982; Welsh et al., 1986). A number of case reports describe bipolar subjects that show 48hr cycling not only in mood but also sleep length, with manic days and short sleep alternating with depressed days and long sleep bouts (Wehr et al., 1982; Welsh et al., 1986; Wilk & Hegerl, 2010). Additionally, sleep-

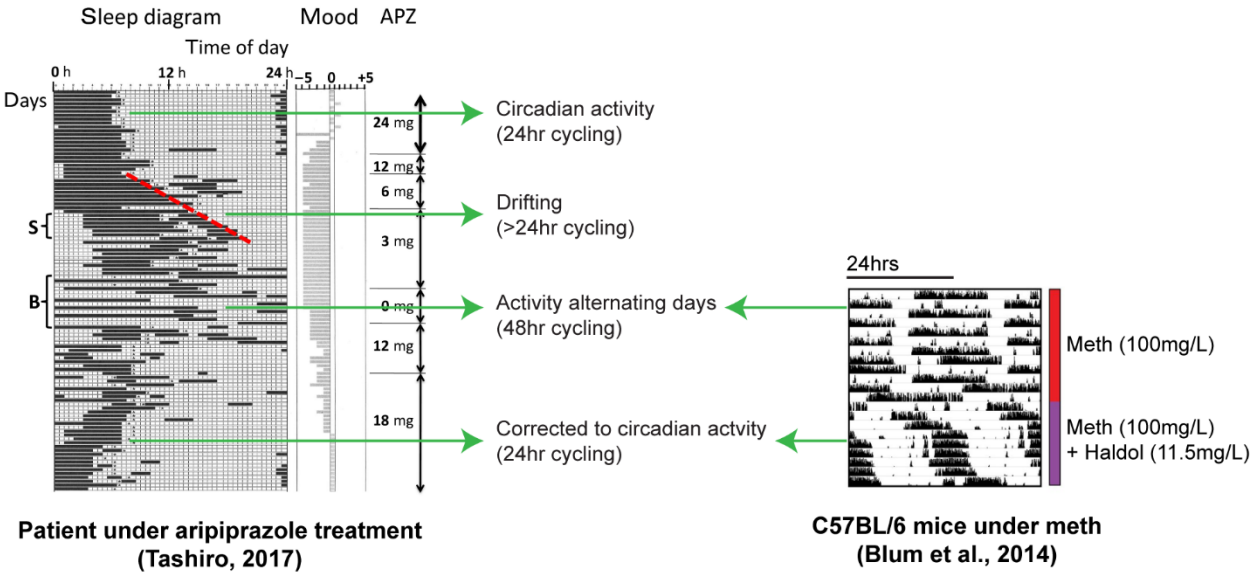


Figure 1.2 : Period correction of infradian rhythms by antipsychotics in human and mouse

Left: Antipsychotic aripiprazole de-/repletion in a BD patient resulting in the appearance and disappearance of infradian rhythms, respectively.
Right: Addition of the antipsychotic to Meth containing drinking water results in period shortening of 48hr cycling mice towards a circadian periodicity

wake pattern of the drifting type has been also reported in bipolar disorder (BD), where sleep onsets and phasing seem to gradually shift to later hours across successive days, reminiscent of what has been described for N24SWDS (Wehr et al., 1998).While 48hr

rhythmicity in arousal causes a misalignment of wakefulness with the circadian clock every other day, patients with longer than 24hr drifting sleep-wake rhythms also become misaligned with the circadian clock as the daily sleep bout drifts into the dark period. This periodic re-/misalignment may concurrently also affect mood resulting in equivalent cycles of mania and depression (Wehr et al., 1982; Wilk & Hegerl, 2010).

When mice are exposed to Methamphetamine chronically, their sleep-wake pattern changes drastically. They exhibit both drifting and 48hr cycling rest-activity patterns that resemble the sleep-wake pattern in patients with BD (Blum et al., 2014; Bourguignon & Storch, 2017). Notably, antipsychotics that are used as the primary treatment for relieving the symptoms of mania in bipolar disorder (Garfinkel et al., 1980) also correct the Meth-induced infradian sleep aberrations in mice (Blum et al., 2014). Intriguingly, a similar 'correction' effect was recently also observed in a BD subject. It was shown that a gradual decrease in the dosage of the antipsychotic aripiprazole unmasked or unleashed an infradian sleep-wake rhythms in the patient with BD, while the subsequent reintroduction of the drugs reverted the rhythms aberrations (Fig. 1.2, adapted from (Tashiro, 2017)). A similar correction effect has also been demonstrated in mice with Meth-induced infradian rhythms that additionally received the antipsychotic haloperidol in the drinking water (Fig. 1.2, adapted from (Blum et al., 2014)). Both antipsychotics as most other members of this drug class have substantial affinities to the DRD2 receptor (Leysen et al., 1992; Shapiro et al., 2003) representing a possible lead towards the molecular mechanism of the rhythms correcting effect.

Collectively, these results indicate that the aberrant rest-activity patterns observed in the above-described mouse models and some patients with BD are strikingly similar pointing

to a shared underlying cause, such as a dopaminergic oscillator operating in the infradian range

1.5 Thesis objectives and organization

This thesis is centrally based on the previous evidence for the existence of a dopamine-based oscillator that regulates sleep-wake cycle independently of the circadian clock. Until this study, rhythms of arousal were largely defined to be controlled by an interaction between the sleep homeostat and the circadian clock and under certain circumstances, by a second rhythmic arousal process, the MASCO, in the presence of Meth. The location, properties and the mechanistic basis of the MASCO remained largely unknown until the discovery of the DUO. It was found that the activity rhythms that manifested upon exposure to Meth could be manipulated by changing the levels of DA, either by changing the Meth concentration in the drinking water or by disrupting the DAT gene. While these manipulations leading to infradian rhythm generation has advanced our understanding of the underlying oscillator, we still do not understand how this oscillator generates rhythmic DA, regulates activity alongside the circadian clock and where it resides. The aim of this thesis is to provide evidence for the neural substrate that plausibly contains the DO and some further insights into the molecular players that are critical for DO rhythm generation. To this end the presented work utilized targeted loss of function approaches to pinpoint the exact location of this 2nd sleep-wake oscillator and to advance its molecular characterization.

Chapter 2 provides a detailed description of the methodology related to the experiments in chapter 3, 4, 5, and 6.

In chapter 3, we build upon previous studies of the DO tunability in mice that lack circadian clock and extend it to the mice that have a functioning circadian clock. We demonstrate that the DO can also adopt a wide range of periods from 26hrs to 72hrs in circadian clock intact mice, wider than previously reported. We also suggest an additional way of analyzing the degree of the oscillator's ability to adopt infradian periodicities, accounting for the lability and prone of the DO to sudden period changes.

In chapter 4, we employ loss of function approaches to target midbrain dopamine neurons and demonstrate that dopaminergic neurons are necessary for 2ndC induction. We also show that production of DA by those neurons is required for 2ndC induction.

In chapter 5, we demonstrate that the VTA-NAc dopaminergic circuitry possibly harbors the oscillator and is sufficient to induce locomotor period lengthening.

And finally, in chapter 6, we demonstrate that vesicular release of DA by VTA DA neurons is not required for 2ndC induction.

The thesis concludes with a general discussion (chapter 7) and concluding remarks (Chapter 8) entailing the implications and limitations, the presented findings as well as future directions.

Chapter 2: Methodology

2.1 Animals

DAT-Cre mice (Turiault et al., 2007) were used for viral targeting of VTA DA. $Th^{flox/flox}$ mice (Darvas et al., 2014) were used for viral-mediated *Th* disruption and $Slc18a2^{flox/flox}$ mice (Narboux-Nême et al., 2011) for Vmat2 disruption in the VTA. All mouse lines were on a C57BL/6 background. All animal procedures were carried out in accordance with the recommendations of the Canadian Council on Animal Care (CCAC) and have been approved by the Mcgill Facility Animal Care Committee (FACC) at the Douglas Mental Health University Institute .

2.2 Locomotor activity monitoring

Animals were individually housed in running wheel cages in light-controlled cabinets under constant darkness conditions and their activity was continuously recorded using clocklab software (Actimetrics, Wilmette, IL). ClockLab software and in-house Matlab-based GUI was used to generate actograms, wavelets, and Lomb-Scargle periodograms.

2.3 Pharmacology

Stock solution of Methamphetamine hydrochloride (National Institute on Drug Abuse, Drug Supply Program, Bethesda, MD) at a concentration of 100mg/L was prepared using drinking tap water. Meth stock solution was diluted to 25mg/L and supplied to the animals for 3-4 days, subsequently increasing the concentration to 50mg/L for another 3-4 days and then further raising it to 100mg/L, maintaining this concentration in the drinking water for 4-8 weeks. Stock solutions of Haldol (11.25 mg/ml, Sigma-Aldrich) and CNO (15 mg/L, National Institute of Mental Health, Bethesda, MD) were

dissolved using drinking tap water. CNO stock solutions were diluted to 7.5mg/L and supplied to Bmal1KO animals with or without 6mg/L Haldol.

2.4 AAV delivery

Mice were anaesthetized with isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments). Recombinant AAV vectors were bilaterally injected into to the VTA area (coordinates from bregma: AP: −3.44 mm, DV: −4.40 mm, L: ±0.48 mm; through a cannula (33 gauge, Plastics One) at a flow rate of 0.1 µl/min for 3 min (0.25-0.3 µl total volume per side) using a syringe pump (Harvard Apparatus). Mice were subsequently maintained in individual housing for at least 4 weeks prior to CNO treatment and/or locomotor activity recording. Viruses: AAV5-hSyn-GFP-Cre (University of North Carolina (UNC) Vector Core, titer $3.5x10^{12}$ genomes copies per ml, diluted 1:1 with saline); AAV5-Flex-taCasp3-TEVp (9) (UNC Vector Core, titer $4.6x10^{12}$ gc/ml); AAV8-hSyn-DIO-hM3D(Gq)-mCherry (14) (MTP, CERVO Brain Research Center, Laval, titer $7.9x10^{12}$ gc/ml).

2.5 6-OHDA injections

6-OHDA (10ug/ul concentration) was stereotoaxically injected into the medial NAc region (coordinates from bregma: AP: +1.10 mm, DV: −4.40 mm, L: ±0.50 mm), at a flow rate of 0.1 µl/min for 2.5 min (2.5ug per side), equal volumes of saline were injected in the control group. Each animal received 25mg/kg desipramine hydrochloride i.p. 30 mins prior to the surgery to spare norepinephrine neurons from 6-OHDA-mediated neurotoxicity.

2.6 Actograms and wavelet analysis

Running wheel revolutions were plotted with 5 min binning at conventional 24 hr scaled double-plotted actograms for 60 days using Clocklab software. Wavelet ridges were calculated from the continuous wavelet transforms using the Clocklab software and the group data was displayed using heatmaps. Typically, two weeks of data were used to compute periodograms and the group periodogram data was plotted in heatmap format. The dominant period in the periodogram was used as the scale for plotting 60 days modulus actograms using Clocklab software. To assess the effect of Meth on locomotor activity, total daily locomotor activity (LA) was calculated based on 10 consecutive days of data prior to and during Meth treatment. Circadian locomotor period was derived from LS periodogram from 10 days of data prior and during Meth treatment in water. Area under the curve (AUC) analysis of the 'circadian' and infradian' range sections of the periodograms to assess infradian rhythm emergence was carried out using GraphPad Prism. For hyperactivity analysis, the total LA from 10 consecutive days prior to and during Meth treatment were calculated based on running wheel activity counts. The values obtained were then normalized by dividing each by the sum of the totals (total prior Meth + total during Meth).

2.7 Power spectral density (PSD) and infradian component calculations

To determine the strengths of circadian and infradian oscillations, we calculated the sum of amplitude (area under the curve) of all significantly rhythmic periodicities ($\alpha = 0.001$) in the Lomb-Scargle periodogram to determine their power spectral density. Periodicities in the 20-27 hr range were assigned as 'circadian' and periodicities in the 27-96 hr range as 'infradian'. For this analysis, Lomb-Scargle periodogram were computed from locomotor

activity data spanning consecutive days prior and during Meth treatment. The respective circadian and infradian densities were then normalized to the total significant spectral density in 20–96hr range and expressed as percentage PSD to represent the extent of circadian and infradian rhythmicity. The analysis was performed using the AUC analysis in GraphPad.

2.8 Immunohistochemistry

Immunostaining was performed as previously described (Blum et al., 2014). Briefly, mice were deeply anesthetized and transcardially perfused with 10% formalin (Z-fix, Anatech). Brains were postfixed in 10% formalin overnight and then incubated in 20% sucrose in saline for at least 24 hours. Brains were cut at 40 microns using either a cryostat (Leica, Solms, Germany) or vibratome (VT 1200S, Leica, Wetzlar, Germany) and collected in either 3 or 4 series per brain. For immunohistochemistry, sections were rinsed in PBS (pH 7.4), incubated in blocking solution (1% goat or donkey serum in 0.3% Triton X-100 in PBS) for 1 hr, followed by incubation with the primary antibody in blocking solution at 4^0C overnight. Sections were then incubated in secondary antibodies for 2 hours at RT. Sections were mounted on superfrost slides (VWR, Radnor, PA), coverslipped with Vectashield mounting medium with DAPI (Vector Labs, Burlington, Canada) and imaged by fluorescence microscopy. Primary and secondary antibodies were used at the following dilutions: rabbit anti-RFP, 1:1000 (Rockland, Limerick, PA) to enhance detection of mCherry expression, anti-GFP, 1:1000 (Invitrogen, Life Technologies, Carlsbad, CA) for GCaMP6s detection and mouse anti-TH, 1:1000 (EMD Millipore, Etibicoke, Canada) for tyrosine hydroxylase detection; Alexa 488 and Alexa 568 conjugates (Life Technologies) were used as secondary antibodies at a dilution of 1:500.

2.9 In situ hybridization

In situ hybridization was performed as described previously (Kraves & Weitz, 2006) . Briefly, fixed brains collected after intra-cardiac perfusion (Z-fix: 10% aqueous buffered zinc formalin, Anatech LTD, Battle Creek, Michigan, USA) were cut at 25 microns using a cryostat (Leica, Solms, Germany) and stored at −80°C until hybridization. Sections were hybridized overnight at 60°C to a digoxigenin-labeled riboprobe targeting the coding regions of mouse Vmat2 (nucleotides 141–265 of the Slc18a2 mRNA, Genbank, NM_172523.3)

2.10 Statistical analysis

Data are presented as mean ± standard error of the mean. One-way and repeated measures, two-way ANOVA followed by a Sidak/Bonferroni post hoc test, t-test, Mann-Whitney U test analyses were performed using GraphPad.

Chapter 3: Meth and DO tunability

3.1 Introduction

Studies on the effect of Meth on animal's rest and activity rhythms have typically used 0.005% (50mg/L) Meth in drinking water as a standard concentration (Taufique et al., 2022). At this concentration, clock-deficient laboratory rodents typically exhibit locomotor activity rhythms under constant conditions (constant darkness) with periodicities not far from 24hrs (S. Honma et al., 2008; Mohawk et al., 2009). Because of this observation, the Meth-induced activities were considered the output of a circadian range timer and hence the underlying oscillator process was referred to as a Methamphetamine sensitive circadian oscillator (MASCO) (Tataroglu et al., 2006). It was however later shown that when Meth is given at concentrations lower and higher than 50mg/L, respectively, SCN-lesioned or otherwise clock deficient animals can adopt ultradian and infradian rhythmicity outside of what is considered the typical circadian range of periodicities, i.e. 20-27hrs (Fig. 3.1a,b. Adopted from (Blum et al., 2014)). Furthermore, MASCO-like rhythms can also result from DAT gene disruption or chronic activation of midbrain dopamine neurons by chemogenetics, with both interventions also increasing extracellular DA levels (Blum et al., 2014; Giros et al., 1996b). These findings are consistent with the view that a secondary oscillator system must exist that can exhibit periodicities in the ultradian, circadian, infradian range. Furthermore, as periodicities can gradually lengthen from ultradian to circadian and ultimately the far infradian range, the data supports the notion that the oscillator is highly tunable, i.e., it does not exhibit a limit in periodicities it can adopt. Hence it was suggested that the MASCO rhythms represent a particular manifestation of the DO in the circadian range owed to the relatively low Meth

concentration provided in the drinking water (Blum et al., 2014) because increasing Meth concentrations not only increase the DO period but also extracellular DA concentrations which is also the case upon DAT disruption. Thus, it seems likely that either an extracellular increase or the concomitant intracellular decrease of DA (Gainetdinov et al., 1998) are critical for DO period determination.

In contrast to clock-deficient mice, a systematic analysis of DO tunability has not been carried out so far in clock-intact mice.

MASCO/DO seems to manifest itself as a 2nd rhythmic component (2ndC) typically at periods in the 26-30hr range (Taufique et al., 2022), which is likely due to the relatively low Meth concentration used (50mg/L). This is additionally supported by the 2ndC observed in DAT KO mice which also seems to settle in the circadian range (~26-28hrs) (Blum et al., 2014). However, there have been two studies reporting 48hr rhythmicity when the Meth concentration was doubled to 100mg/L (Blum et al., 2014; Cuesta et al., 2012). This is in line with the effect of 100mg/L concentration of Meth on locomotor rhythms in circadian clock-deficient mice (Blum et al., 2014) suggesting that even circadian intact mice can adopt far-infradian rhythmicities. Furthermore, prolonged exposure of clock-deficient mice to 100mg/L Meth, was found to increase locomotor activity periods in individual animals to 100hr and above. (Fig. 3.1c; (Blum et al., 2014)).

In this chapter, we will explore the tunability of the DO, i.e., the range of periods it is able to adopt in the presence of the circadian oscillator and demonstrate that the Meth-induced 2ndC frequently adopts periods even longer than 48hr when animals are exposed to 100mg/L for extended periods of time. We believe that while the presence of the circadian timer may slow the period progression of the 2ndC under this high Meth concentration, it

will ultimately not be able to halt it. We also partly address the sudden changes in the periodicity of 2ndC as has been mentioned previously (Taufique et al., 2022) and suggest an analysis model to quantify the extent of labile DO rhythmicity in the infradian range.

3.2 Experimental design

All animals were individually housed in running wheel cages for activity recording in constant darkness. Animals in the Meth group used for this chapter are control groups

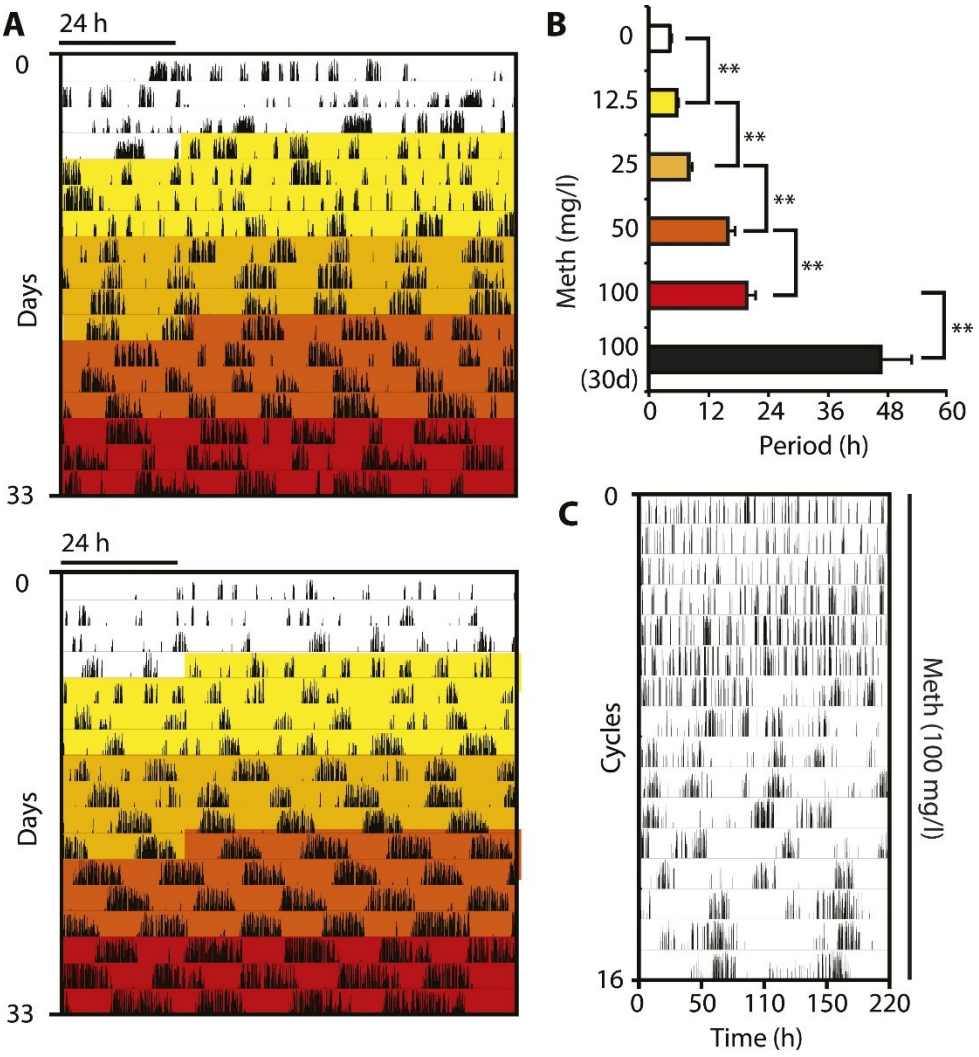


Figure 3.1 : Evolution of ultradian activity during Meth treatment

a) Representative actograms of circadian disrupted Bmal1KO mice under Meth treatment, **b)** Increasing mean periods at increasing Meth dosage, **c)** Modulo 110-hr actogram of a Bmal1KO mice after prolonged exposure to 100mg/L Meth. Adapted from Blum et al., 2014

from several experimental cohorts combined, both males and females included. All animals were injected with saline either in the VTA or NAc. All animals went through a

similar protocol of incremental Meth administration starting at 25mg/L followed by 50mg/L, only for a few days each, and then maintained at 100mg/L for a prolonged duration. The actogram and the continuous wavelet transforms (CWT) include a 10-day baseline prior to the Meth administration.

For Area under the curve (AUC) analysis, n=13 animals from two Meth cohorts and n=12 from animals which received regular water were used. Briefly, power spectral density (PSD) computation of significant rhythmicities as well as area under the curve (AUC) analysis was based on Lomb-Scargle (LS) periodograms derived from 14days of locomotor recordings prior to and during Meth treatment. Highest peak analysis was performed from the same LS periodogram using the AUC method. All analyses were performed on Graphpad Prism software.

3.3 Results

3.3.1 Periodicities of infradian locomotor rhythms under Meth

We compiled the control groups from several experimental cohorts all of which were supplied with Meth via drinking water at incrementally increasing concentrations up to 100mg/L (Fig. 3.1a) and then maintained at that dose for at least 4 weeks. Locomotor

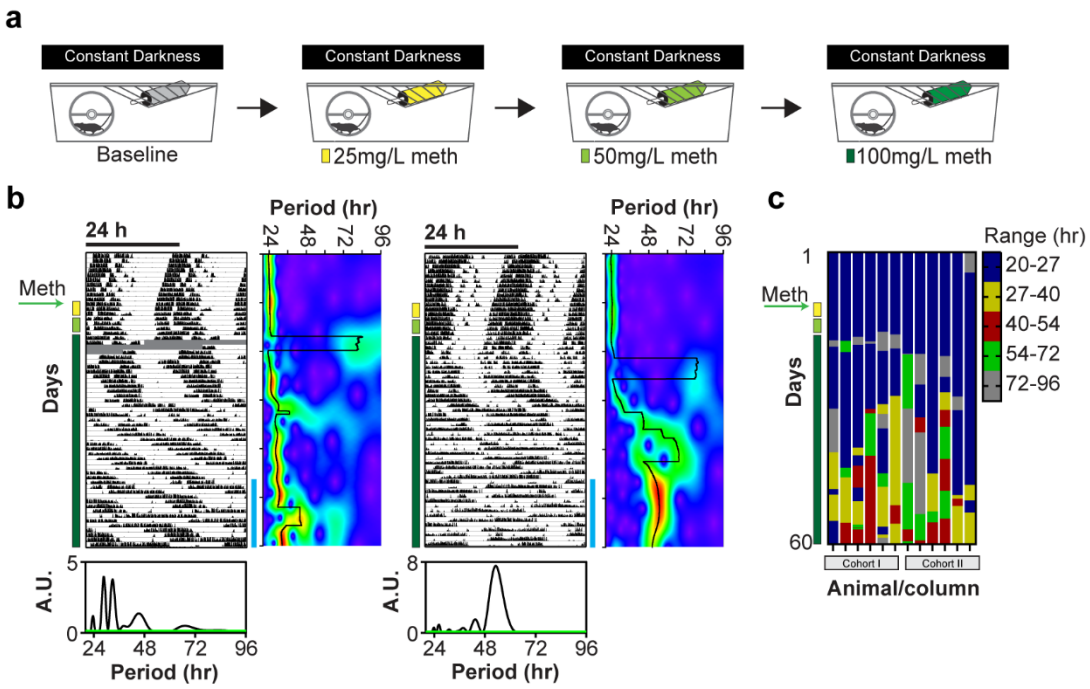


Figure 3.2 : Evolution of infradian locomotor periodicities in response to high concentrations of Methamphetamine

a) Schematic of experimental design to incrementally introduce Meth, at escalating concentration. **b)** Representative actograms displaying running wheel activity patterns single housed animals (Grey colored block in the actogram represents missing data). Continuous wavelet transforms heatmaps next to the actograms show period amplitudes; ridge trace (black) indicates the local amplitude maxima. Below actograms are the Lomb-Scargle periodogram displayed, which were generated from the last 14 days of the displayed running wheel recording (blue bar). **c)** Heatmap displaying group data of the wavelet transform ridges of 2 cohort of mice binned in different periodic ranges represented by different colors (n=13). The number of recorded days and Meth treatment regimen corresponds to the actograms shown in b.

activity was monitored via running wheel counts to assess changes in rest-activity rhythms in DD.

The two Meth cohorts were used to illustrate the evolution of the activity rhythm under 100mg/L Meth and were compared to water treated animals. As expected, animals in the water-only group displayed highly circadian rest and activity patterns (Suppl. Fig. 3.1), while Meth treated animals exhibited distinct changes in the activity patterns within a few days after the drinking water was supplemented with Meth (Fig. 3.2b). The representative actogram on the left shows a 'drifting only' phenotype characterized by consecutive delays in the onset of activity (towards the right of the actogram), which is due to the 2ndC exhibiting periodicities that are longer than 24hrs. The animal on the right however shows this drifting behavior only initially, morphing into a pattern with activity bouts shown every other day. This pattern of evolution is consistent with the emergence of a 2ndC that first adopts periods slightly longer than 24 and then further lengthens to a period of 48hrs. The period changes in the rest-activity patterns are further demonstrated by the CWT heatmaps and their ridge traces next to the actograms. The ridge plots illustrate the gradual evolution towards longer than 24hr periodicities indicative of the emergence of an infradian 2ndC (Fig 3.2b). The Loomb-Scargle periodogram below the actogram further corroborates the presence of non-24 hr infradian periodicities under the 100mg/L Meth regimen (Fig 3.2b).

The evolution of the locomotor period for two Meth cohorts are shown in the composite CWT heatmap showing binned values of the ridges which trace the dominant period (Fig 3.2c). The heatmap display demonstrates considerable onset variability in infradian rhythmicity emergence as indicated by the different lengths of the blue color bars, which

reflect 20-27hr periodicities and thus time spans where the circadian rhythmic component dominates (Fig 3.2c).

3.3.2 A new method for assessing the extent of infradian rhythm expression

The LS periodogram shows the expected dominant circadian peak in a mouse that receives tap water only (3.3a), however multiple substantial infradian peaks emerge in a mouse with Meth supplemented to the drinking water (Fig 3.2c). This periodogram pattern is frequently observed under exposure to a high concentration (100mg/L) of Meth during the time span used to compute the periodogram. Due to the labile nature of the DO, its periodicity can substantially change over the course of a few days, which in turn results in multiple peaks in the periodogram when the time span used to compute the periodogram is long enough as is typically the case for a 14day period as used here. The composite heatmap display of the periodograms from animals treated with water only or Meth further corroborates the occurrence of multiple infradian peaks upon Meth treatment (Supp. Fig. 3.2).

The period of an oscillator is usually defined by the highest periodogram peak, yet due to the intra-animal period variability upon Meth exposure which often results in multiple infradian peaks, the highest peak may not represent the dominant rhythmicity. To address this issue, we propose the sum of amplitudes of oscillations i.e., the total PSD for a given range as an additional metric to measure circadian and infradian rhythmicity. Since the PSD is normalized for each periodogram, the extent of periodicities among different animals can be compared. For the analysis, the sum of amplitudes across the 20-27hr period range, which equals the PSD for that range, was computed as measure of circadian rhythmicity, whereas the sum of amplitudes across the 27-96hr represented the

measure for infradian rhythmicity. We then calculated the percentage contribution of each of the two bins to the total PSD stretching from 20-96hr. This quantitative analysis confirmed that circadian rhythmicity dominated in the water only treated animals with almost 100% of the total PSD allocated to the 20-27hr range (Fig. 3.3b). In the Meth-treatment group, on the other hand, the inverse was observed, most of the total PSD was

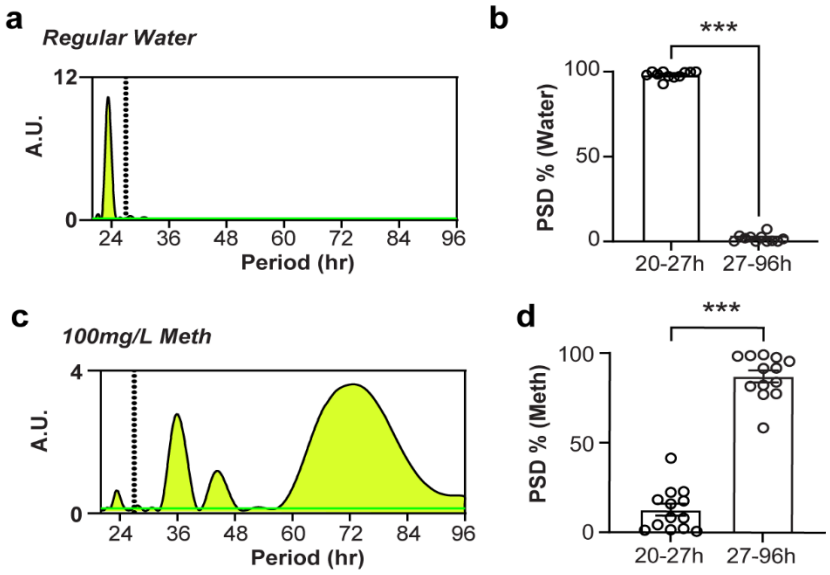


Figure 3.3 : Area under the curve analysis.

a) LS periodogram during water only treatment computed from 14 days of locomotor recordings, dotted line divides circadian [20-27hr] from infradian ranges [27-96hr], **b)** Periodogram derived normalized PSD (area under the curve) of water-only treated group, circadian vs infradian range. **c)** 14 day-based LS periodogram during 100mg/L Meth (n=13) **d)** normalized PSD of Meth-treatment group, **d)** circadian vs infradian range. Group data are means±SEM. Two-way ANOVA with Sidak's comparison test. ***P<0.001.

allocated to the 27-96 hr range (Fig. 3.3d) supporting the profound presence of infradian rhythmicity and a reduction in circadian activity. Notably, the number and sizing of the period range bins can be altered too, for instance to determine if specific infradian

frequencies are preferentially adopted by a 2ndC that emerges upon e.g., a specific genetic manipulation.

3.3.3 Wide tunability of the DO in circadian-intact mice at high concentrations of Meth

In order to determine if a high concentration of Methamphetamine in the drinking water indeed lengthens the Meth-dependent 2ndC well into the infradian range in mice with an

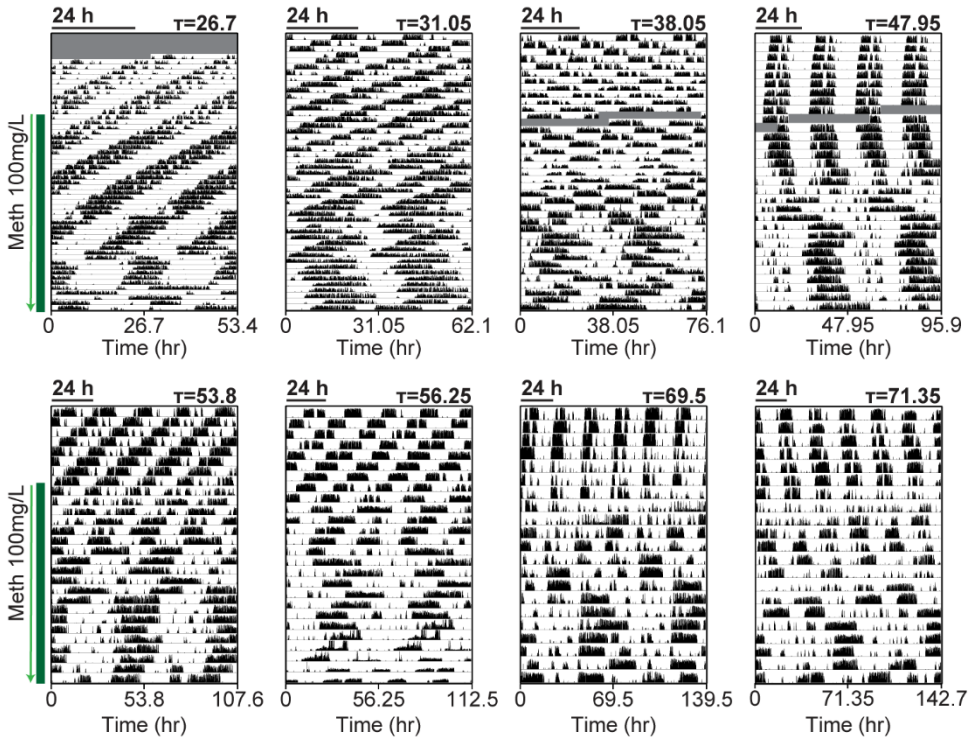


Figure 3.4 : Modulus actograms of animals with various periodicities

Representative actograms from circadian-intact mice exposed to 100mg/L Meth. Actograms are ordered by the modulo period derived from the highest peak of the periodogram computed from the last 14 days of the 60-day actogram. The modulus actogram demonstrates rhythmic activity in the near to far infradian range.

intact circadian timer, we exposed them to 100mg/L for extended periods of time. The running wheel activity data was then plotted in actogram format, however the extent of the x-axis was determined by the dominant locomotor period the animals adopted during the last 14 day of the 60 days actogram. This period was derived from the highest peak

in the respective periodogram computed for this time span. The resulting representative actograms visually confirmed that the 2ndC can reach periodicities far into the infradian with single animals exhibiting period lengthening up to 72 hrs under 100mg/L Meth (Fig. 3.4).

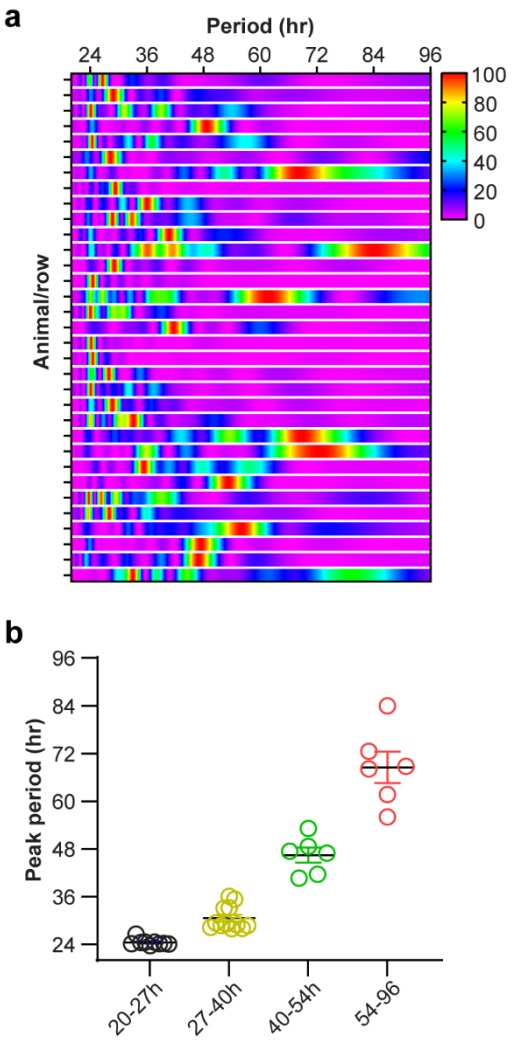


Figure 3.5 : Dominant rhythmicity of sleep-wake rhythms.

a) Composite heatmap of 14 days LS periodogram of all the control group animals with Meth, each row represents an animal, **b)** Periodogram derived highest peak of oscillations of each animal binned in different period ranges from 20-96hr (n=33). Error bars indicate mean and SEM, **c)** Scatter plot of highest peak oscillation of each animal against its own infradian PSD%

Fig, 3.5a shows the periodograms computed from the last 14 days of the exposure to 100mg/L Meth of all cohort animals. The spectral energy displayed in the heatmap demonstrates a wide range of periodicities with most animals displaying substantial energy in the infradian range in addition to the circadian rhythm energy which varied in strength. As alluded above, the occurrence of multiple peaks in the infradian range is likely owed to the relative period instability of the DO versus the circadian timer. Notably, some animals show a lack or great attenuation of the circadian energy indicating that running wheel activity in these cases is predominantly controlled by the DO and not any longer by the circadian timer. Despite the presence of multiple strong peaks in the majority of the animals, a considerable number showed just 1 major infradian peak in addition to the circadian peak, consistent with two oscillator control of locomotor activity in the presence of Meth (Fig 3.5a). The periods at the highest peak per each periodogram were then distributed in 4 bins, reflective of the circadian (20-27hr), the near infradian (27-40hr), mid infradian (40-54hr), and far infradian (54-96hr) range (Fig 3.5b). While with regard to infradian ranges, the near infradian range dominated somewhat, the graph clearly reveals that for a considerable number of animals (36%), the dominant periodicity falls into the mid to far infradian range, i.e., show periodicities of >40hrs.

Notably, some animals exhibited locomotor activity rhythms with periodicities in the 54-96hr range (see heatmap in Fig.3.5a and 54-96hr bin in Fig.3.5b and heatmap) which have never been reported before for circadian intact animals under chronic Meth treatment.

3.4 Conclusion

While the circadian clock especially in the absence of a light:dark cycle chiefly shapes the daily pattern in rest and activity in rodents, under certain conditions, its rhythmic control is overtaken by an independent secondary oscillator process which is likely dopaminergic in nature, thus the name dopaminergic oscillator (DO), also referred to as MASCO by other groups (Tataroglu et al., 2006).The DO seems to have no limits with regard to the infradian periods it can adopt. Rhythms of 100+hrs have been observed under the specific condition of Meth treatment in constant darkness and absence of an endogenous circadian timer (Blum et al., 2014). These data clearly indicate a high degree of tunability of the DO oscillator in clock-deficient mice. However, in circadian intact rodents, which were the subject of most studies exploring the effect of Meth on 2ndC emergence, their 2ndCs have been found to typically fall into 26-30hrs period range. As most previous studies however used a rather moderately high concentration, 50mg/L (0.005%) of Meth to induce and maintain the 2ndC. In this chapter, we provide evidence that the low Meth concentration used in previous studies is likely the culprit why Meth-induced rhythms hovered at 26-30 hrs, not far from the 24hr periodicities produced by the circadian timer. We not only demonstrate that a substantial number (>30%) of circadian intact animals treated with higher concentrations of Meth (100mg/L) produce dominant rhythmicities with period longer than 40hrs, but that some animals (~20%) even exhibit periodicities beyond 54hrs which has thus far not been reported. The finding further confirms that DO's tunability is dependent on the Meth concentration in the drinking water and thus the DA levels. The higher the concentration of Meth, the longer the periodicities the animals can achieve. However, it is still not clear as to why prolonged exposure of Meth causes further

increase in the period of the oscillator (see evolution of periodicities over time in Fig. 3.2c). Meth has different levels of actions, from inhibiting DAT-mediated dopamine re-uptake and promoting DA efflux by DAT action reversal to unpackaging of vesicular dopamine by reversing the vesicular Vmat2 transporter (Seiden et al., 1993; Sulzer et al., 2005a). It can be postulated that during chronic Meth exposure, dopamine from so called reserve vesicular pools is set free to further the increase in dopamine tonality (Seiden et al., 1993) which in turn drives period length. It is conceivable that perhaps this DA source is not available at lower (25-50mg/L) Meth concentrations or when Meth is provided for only relative short periods of time.

Notably in this context, we found cage changes to represent a substantial stressor for an individually housed animals. Cages changes have been reported previously to cause sudden period changes in Meth treated animals, which usually occurring right after a cage change occurred (Taufique et al., 2022). To mitigate the risk of cage-change induced period changes, which mostly had the directing to shorter periods, we started to transfer a small amount of used bedding including feces to the new cage and found this measure to completely prevent cage change related period changes. In addition, we extended the period between changes from 2 to 3 weeks to maximize the time span of undisturbed behavioral activity. These methods together contributed to minimizing ‘fall backs’ to shorter periods and thus critically helped to enable the 2ndC to adopt far-infradian periodicities of 48hrs and beyond. However, as demonstrated by the ridge plot in Fig.3.2b, despites these measures, DO activity is still characterized by more or less strong degree of period instability across time. The DO oscillator can seemingly adopt a new period from one day to the other that can deviate from its previous period by hours to even days (see

Fig.3.2b). This behavior seems the chief reason why a periodogram computed from 14 consecutive days of locomotor activity shows multiple peaks in the infradian range. It is not due to multiple infradian oscillators but rather the consequence of a single oscillator (the DO), that is period labile. Due to this 'artifact' we adopted the AUP calculus for a quantitative assessment of infradian range rhythmicity as this takes infradian (and circadian) energy in its entirety into account. This mode of calculating PSD for a given period range corroborated that animals under Meth exhibit a high degree of infradian PSD compared to water-only treated animals (see supplementary Fig. 3.2). This modus of PSD calculation seems to be able to serve as a useful metric to quantitatively assess the degree of infradian rhythmicity or in other words to assess the ability to produce an infradian 2ndC that controls sleep wake rhythmicity.

To conclude, we confirm that the DO is highly tunable even in the presence of the circadian oscillator and its period can be lengthened to at least 72 hrs or 3 days. We also suggest an additional metric to assess the dominance of rhythmicity between the circadian and the dopaminergic oscillator.

Chapter 4: Locating the DO

4.1 Introduction

The finding that DAT disruption leads to the emergence of a 2ndC suggests that DA neurons that express the DAT are likely key substrate of the oscillator process driving the 2ndC. Underscoring this view is the observation that chronic chemogenetic activation of DAT expressing midbrain DA neurons, more specifically DA neurons of the VTA/SN region results in period lengthening of ultradian rhythms (Blum et al., 2014). The later result further suggests that midbrain DA neurons are the key players in DO rhythm generation. While both VTA and SN regions are known to be neuronally active during the wake state (Brown et al., 2012), recent work on the VTA DA neurons extends its critical role in the maintenance of wakefulness as well as ethological sleep regulation (Eban-Rothschild et al., 2016a) strengthening its argument for harboring a sleep-wake regulating oscillator.

In the previous chapter, we introduced a new quantitative method to assess the extent of circadian and infradian rhythmicity and provided evidence that the periods the DO can adopt are variable but are not limited to the circadian or near infradian range. In this chapter, we are investigating the role of VTA DA neurons in rhythm generation by loss of function approaches and using the quantitative methods described above to assess their contributions to Meth-mediated 2ndC emergence .

4.2 Experimental design

We injected an adeno-associated virus carrying a Cre-activatable Casp3 gene bilaterally in the VTA of DAT-Cre mice to induce apoptosis as a consequence of Casp3 expression-

specifically in the DA neurons of the VTA (Fig 4.1a, left). For the selective disruption of tyrosine hydroxylase (TH), an enzyme essential for DA biosynthesis, in the VTA DA neurons, we injected an AAV, which expresses GFP-tagged Cre recombinase, bilaterally into the posterior VTA region of adult $TH^{fl/fl}$ mice (Fig. 4.2a, left). The control groups in both experiments received VTA injections with saline. The injected animals were individually placed in running wheel cages and locomotor activity was monitored while the animals were exposed to incrementally increased concentrations of Meth in their drinking water up to the final concentration of 100mg/L Meth, at which they were maintained for at least 4 weeks before brains were collected for immunohistochemical inspection.

Animals with partial Casp3 mediated ablation or Th disruption based on TH immunostaining in the VTA were not included for the analysis. A few animals that didn't survive during Meth administration were also excluded.

4.3 Results

4.3.1 Selective targeting of VTA DA neurons

The extent of Casp3 mediated VTA ablation upon AAV-DIO-Casp3 injection in the animals was assessed by the absence of TH staining in the midbrain, which is a marker for DA neurons. In the Casp3 injected group, VTA DA neurons were preferentially lost while the DA neurons of the adjacent substantia nigra and the anterior VTA were largely preserved (Fig 4.1a, right). This is likely due to poor Cre activity, as we found reporter-negative TH+ cells preferentially in this VTA region when assessing the fidelity of this DAT-Cre line (Suppl. Fig. 4.1a), as well as a similar retention pattern of TH expression in DAT-Cre x THfl/fl mice (Supp. Fig. 4.1b).

The conditional disruption of the Th gene in the posterior VTA upon injection of AAV-GFP-Cre was also confirmed by the absence of TH staining (Fig. 4.2b, right) and the presence of punctate GFP expression in the VTA, reflective of the GFP-Cre fusion protein's nuclear localization (Suppl. Fig. 4.2). Unlike in the case of Casp3 ablation, there was no retention of TH signal in the ventro-medial VTA upon AAV injection, as the AAV would express Cre indiscriminately across all neurons of the posterior VTA. However, as in case of the AAV-Casp3 approach, the SN was also largely spared upon this manipulation (Fig. 4.2b, right).

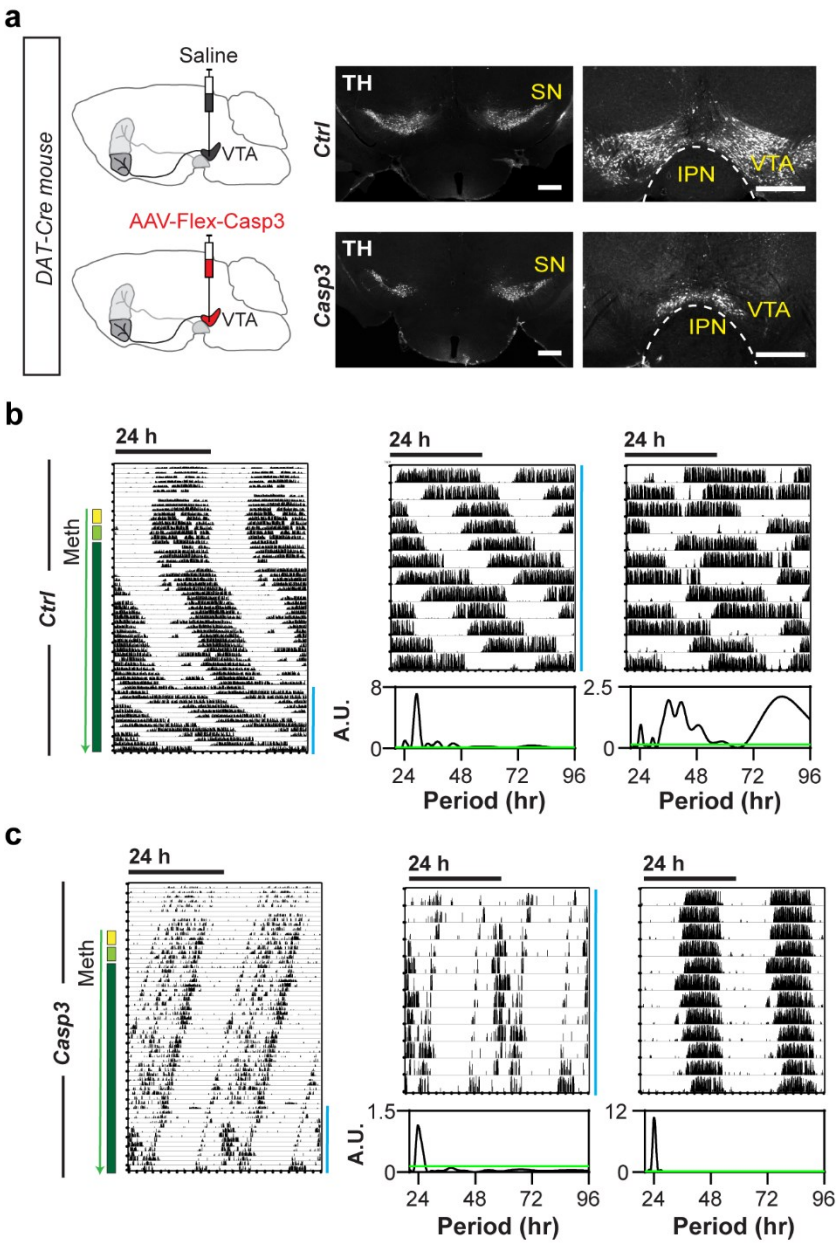


Figure 4.1: Meth-mediated infradian rest:activity pattern is abolished upon VTA DA neuron ablation.

a) Left, schematics illustrating injection of either saline or an AAV expressing Cre-activatable Casp3 in the VTA of *DAT-Cre* mice. Representative immunolabeled coronal sections from SN and VTA are shown on the right. Absence of TH staining in the VTA after AAV-Casp3 injection indicates targeted loss of VTA DA neurons.

b,c) Representative 60 days actograms on the left displaying running wheel activity patterns of control and Casp3 mice in constant darkness in response to 25, 50, and 100mg/L meth (yellow, light green, dark green, respectively) in drinking water. Right, replot of final 14 days of locomotor activity as indicated by blue bar along with an additional representative actogram, LS periodograms are shown underneath.

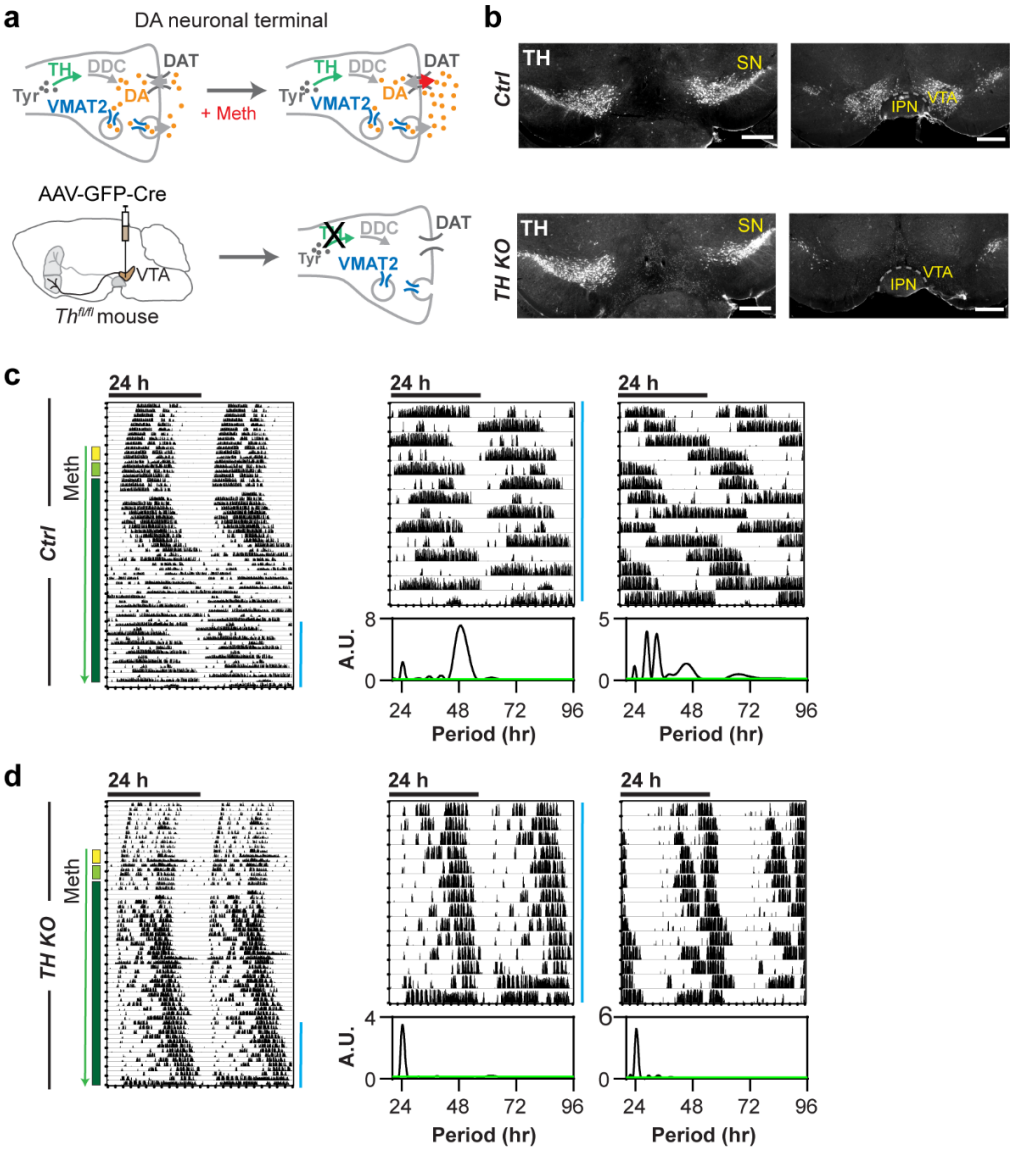


Figure 4.2 : Selective disruption of Th in the VTA leads to loss of Meth-mediated emergence of infradian activity

a) Top row: Schematic illustrating the Meth-mediated increase in extracellular DA levels at the DA terminal. Tyr, tyrosine; DDC, L-dopa decarboxylase. **Bottom row:** Bilateral injection of AAV-GFP-Cre in the VTA of *TH*$^{fl/fl}$ mice resulting in loss of DA from the VTA DA neuronal terminals. **b)** Representative immuno-labeled coronal sections of the SN and VTA region. Absence of TH staining in the VTA demonstrates successful disruption of the Th gene in ^{VTA}THKO animals while the SN and anterior VTA are spared as shown in the lower left. **c,d)** Representative 60 days actograms on the left displaying running wheel activity patterns of control and THKO mice in constant darkness in response to 100mg/L Meth (dark green) in drinking water. Right, replot of final 14 days of locomotor activity as indicated by blue bar, along with an additional representative actogram. LS periodograms are shown underneath.

4.3.2 VTA DA loss abolishes Meth-mediated rhythmicity but not Meth-mediated hyperactivity

We next assessed the effect of Meth on the manipulated animal's rest:activity rhythms. As shown by the representative actograms, exposure to Meth (100mg/L) resulted in the appearance of distinct infradian locomotor activity rhythms in the control animals (Fig. 4.1b, 4.2c). However, ablation of DA neurons in the posterior VTA (Fig. 4.1c) or TH disruption in these neurons (Fig. 4.2d) resulted in loss of Meth-mediated infradian rhythm induction. The two further representative actograms on the right in Fig. 4.1c and Fig. 4.2c respectively, which show only the final 2 weeks of the Meth treatment regimen (indicated by light blue bar) additionally confirm the 2ndC induction capacity loss in the AAV-injected animals. The LS periodograms plotted below the actograms in Fig. 4.1c and 4.2c confirm that the spectral power in ^{VTA}Casp3 and ^{VTA}THKO animals was limited to circadian periodicities throughout the Meth regime, whereas control animals showed the expected infradian spectral energy in response to Meth as reflected by locomotor bout drifting and 48hr bout cycling behavior as demonstrated by the actogram displays (Fig. 4.1 c, and Fig. 4.2 c)

We next assessed total daily running wheel activity 10 days prior to the Meth treatment and during exposure to 100mg/L Meth. The loss of the posterior VTA DA neurons (^{VTA}Casp3) or the loss of their ability to produce DA (^{VTA}THKO) both resulted in the reduction of the total daily running wheel counts during both time spans of water-only and Meth treatment, respectively (Fig. 4.3a,c). These results indicate that posterior VTA-derived dopamine critically contributes to daily locomotor drive and thus likely daily

arousal, or wakefulness and that psychostimulant treatment is unable to fully remedy this deficit.

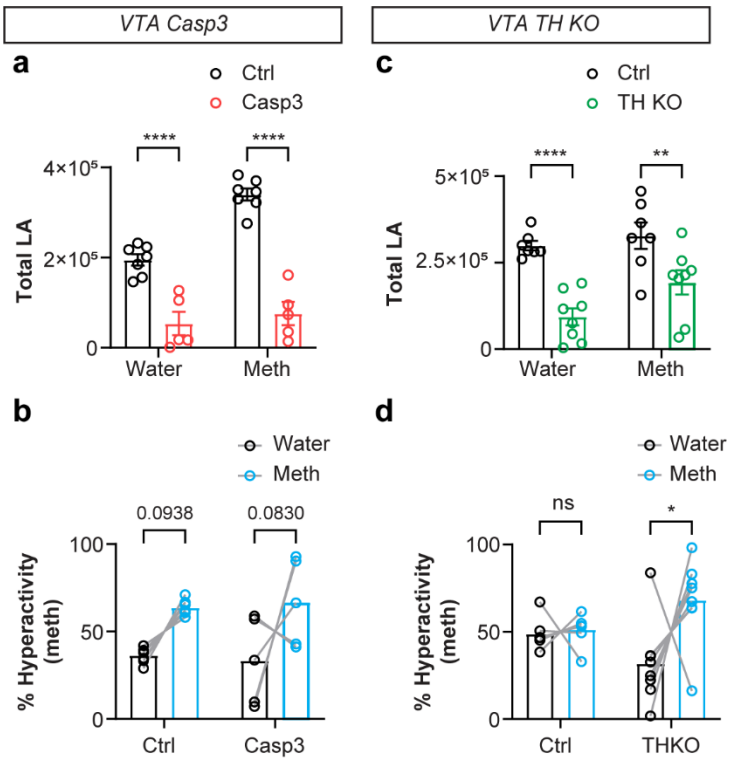


Figure 4.3 : Preservation of Meth-induced hyperactivity in ^{VTA}Casp3 and ^{VTA}THKO mice

a,c) Daily locomotor activity average of 10 successive days of Casp3 and THKO mice compared to their respective controls prior to Meth and during 100mg/L Meth. Group data are means±SEM. Two-way ANOVA with Sidak's comparison test. ns, not significant, *P<0.05, **P<0.01, and ****P<0.0001. **b,d) %** Meth-induced hyperactivity in Casp3 and TH KO mice alongside their controls indicating increased locomotor activity in response to Meth (100mg/L).

Next, we assessed whether Meth-induced hyperactivity was affected by the loss of VTA DA. neurons or their ability to produce DA. We found that even though ^{VTA}TH disruption or VTA DA neuron loss decreased the total daily locomotor activity of the animals, they still exhibited Meth-induced hyperactivity (Fig. 4.3b,d). Both Casp3 (Fig. 4.3b) and THKO

(4.3d) animals tended to show responsiveness suggesting that DA in the VTA may not be necessary for Meth-induced hyperactivity.

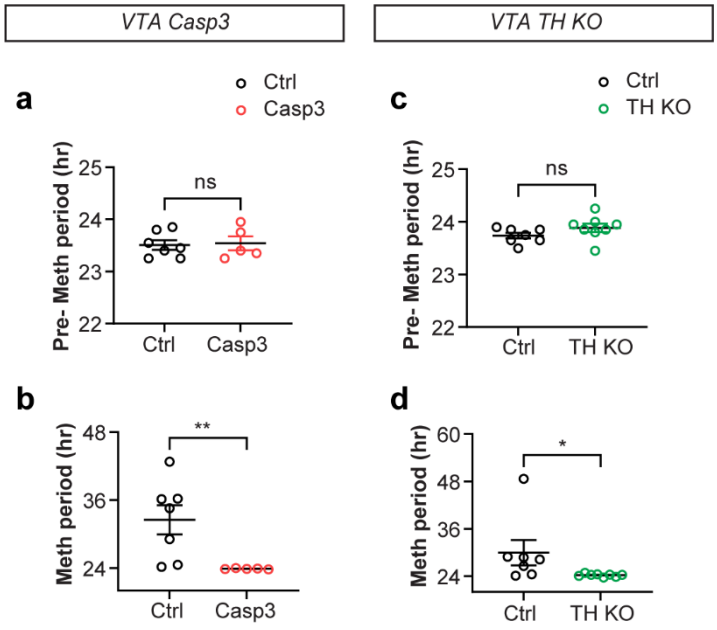


Figure 4.4 : Locomotor period of experimental and control animals prior and during Meth exposure

a,c) Locomotor periods were derived from the position of the highest periodogram peak prior to Meth in both Casp3, THKO animals compared to their controls. Periodograms based on 10d of consecutive running wheel data **b,d)** Locomotor periods in the final 14 days of treatment with 100mg/L Meth. Group data are means±SEM. Unpaired non-parametric t-test with Mann Whitney U test. ns, not significant, *P<0.05, **P<0.01,

To assess if VTA DA neuronal ablation or ^{VTA}TH disruption affects circadian rhythmicity per se, we plotted the period of the highest peak from the 20-96hr Lomb-Scargle periodogram prior and during Meth. Neither prior to (Pre-Meth) nor during Meth treatment, both Casp3-mediated ablation of VTA DA neurons and the VTA specific TH KO seem to affect the circadian control over locomotion as evidenced by the fact that the highest periodogram peak retained its position close to 24hr (Fig. 4.4a-d). As expected, in the

control animals the highest peak periodicity tended to shift into the infradian range upon Meth treatment due to the DO more or less ‘taking over’ rhythmic control of the daily rest:activity pattern (Fig. 4.4b,d). Notably, a number of control animals showed only a slight shift into the infradian or no shift at all (see particularly Fig. 4.4d). This is likely due to the fact that (DO controlled) infradian rhythmicities show period lability as opposed to circadian clock controlled rhythmicities, which leads to much broader and/or multiple infradian peaks with none becoming the highest peak of the 20-96hr periodogram range and thus the circadian peak may still retain its highest peak status despite profound infradian rhythmicities.

Because of this, we adopted the AUC-based quantification of PSD to assess infradian rhythmicity versus circadian rhythmicity in ^{VTA}Casp3 and ^{VTA}THKO mice. We found that prior to Meth, both, ^{VTA}Caps3 and ^{VTA}THKO mice showed no significant differences in circadian vs. infradian range distribution of PSD when compared to their respective controls (Fig. 4.5a,d). However, during Meth treatment, as expected a large portion of PSD shifted into infradian range (27-96hr) in controls with PSD in circadian range (20-27hr) proportionally decreasing while the ^{VTA}Casp3 and ^{VTA}THKO showed no significant shift towards the infradian range and thus retained a significantly higher portion of the PSD in the circadian range (Fig. 4.5b,e). The composite heatmap displaying the normalized periodograms visually confirms the loss or attenuation of infradian energy and thus rhythmicity induction by Meth in ^{VTA}Casp3 and ^{VTA}THKO mice (Fig. 4.5 c,f).

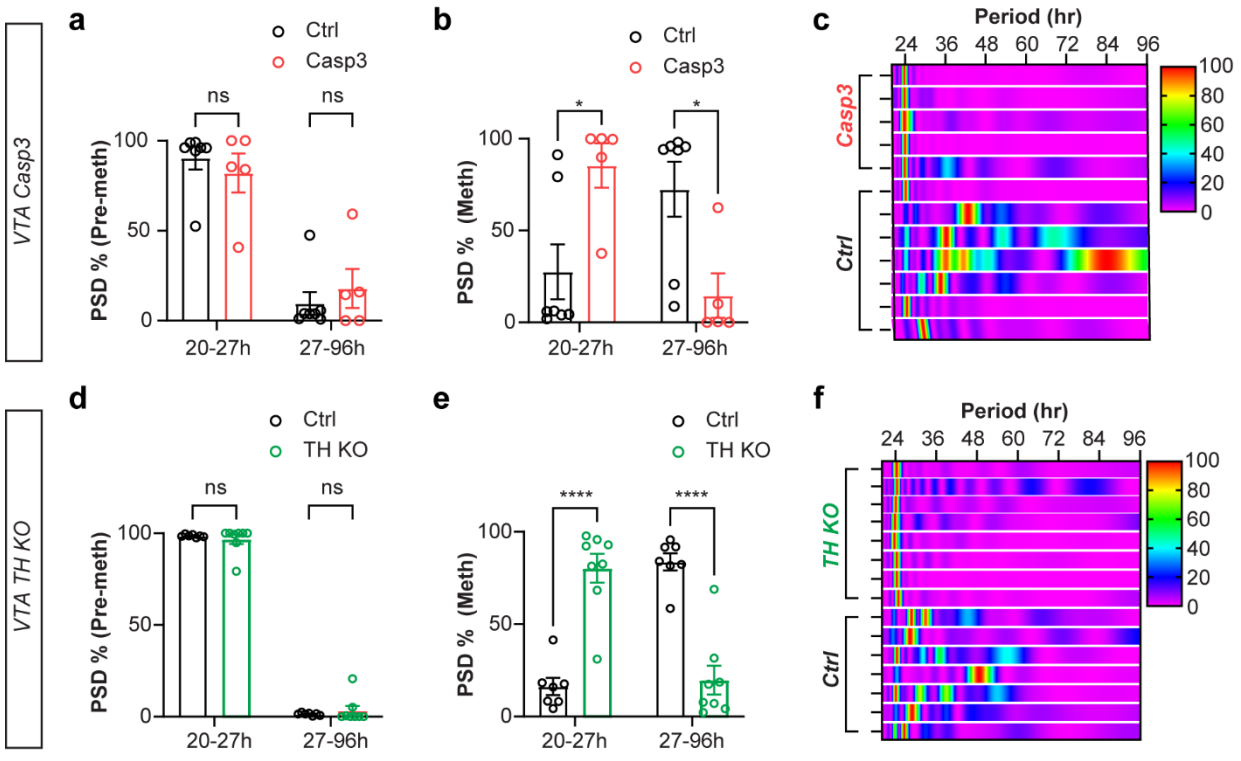


Figure 4.5 : Quantitative analysis of the loss of infradian rhythmicity under Meth in ^{VTA}Casp3 and ^{VTA}THKO mice

a,d) PSD distribution (%) of significant rhythmicities in Casp3 and TH KO mice and their respective controls between circadian 20-27hr and infradian 27-96hr range before Meth and **b,e)** during 100mg/ml Meth treatment. Group data are means±SEM. Two-way ANOVA with Sidak's comparison test. ns, not significant, *P<0.05, ****P<0.0001,
c,f) Composite heatmaps showing normalized periodograms from individual mice during Meth exposure .

Together these results indicate that DA neurons of the VTA and here specifically their ability to produce DA are necessary for infradian rhythm generation.

4.4 Conclusion

In this chapter, we provide the first clear insight into the neuronal substrate that is required for Meth-dependent infradian locomotor rhythm emergence and by extension, the substrate that is needed for DO rhythm production.

By employing specific genetic manipulations targeted towards VTA dopamine neurons, we show that the DA neurons of the posterior VTA are necessary for infradian rhythm emergence. We further show that by disrupting the Th gene across the posterior VTA, it is likely specifically the ability of these DA neurons to produce DA which is necessary for infradian rhythm production. Importantly, both manipulations spared the anterior VTA and the SN, arguing that neither the DA neurons of the SN nor those of the anterior VTA are sufficient to produce a 2ndC in response to Meth. Since these loss of function approaches concomitantly resulted in an overall decrease in locomotor activity, it is tempting to speculate that the loss of the capacity to produce infradian rhythms is a consequence of the overall reduction in LA. A first hint that this might not be the case comes from our observation that Meth is still able to increase overall locomotor activity in the experimental mice (^{VTA}Casp3 and ^{VTA}THKO) indicating that the circuits that produce the hyperlocomotor phenotype upon psychostimulant treatment are still at least partially intact. This finding thus supports the view, that the circuits that facilitate psychostimulant-induced hyperlocomotion are distinct from or at least only partially overlap with the cells/circuits required for 2ndC induction and that the posterior VTA DA neurons are required for 2ndC mergence but at most only partially needed for hyperlocomotion induction.

Moreover, our data also shows that while DA neuron or TH elimination from the VTA leads to overall locomotor loss, this loss did not translate in an overt impairment of the circadian timer, as circadian locomotor periodicity did not differ between experimental and control animals and even Meth treatment did not result in a change of the circadian period of experimental mice. This data thus demonstrates that neither VTA DA neurons nor VTA DA production are required for circadian period determination.

Given the role of dopamine in mediating arousal as part of the ascending arousal pathway (Eban-Rothschild et al., 2016b; Saper et al., 2005; Sulaman et al., 2023), it is tempting to speculated that the DA oscillator which may reside in DA neurons in the posterior VTA, acts as an arousal oscillator via its main output, DA. Thus, it may not impose a sleep as well as a wake signal, but instead, it only produces a wake promoting arousal signal each cycle. This may explain why the DO can drive wakefulness even at a time when the circadian sleep pressure is at its highest. It seemingly overrides circadian sleep drive as it progresses through the animal's circadian rest phase when cycling at periods >24hr. This view is also supported by recent evidence that VTA DA neurons can maintain wakefulness even during high sleep pressure (Eban-Rothschild et al., 2016a), i.e., during the day. Thus, the DO may be able to override sleep pressure due to the fact that it drives the rhythmic release of an arousal factor known to affect sleep need (Eban-Rothschild et al., 2016a). Indeed, our lab has found evidence that during DO-mediated 48hr sleep cycling, the animal's overall sleep need seems reduced. Thus, this secondary oscillator system may have specifically evolved within the DA system due to the fact that DA is able to override both the homeostatic and the circadian control of sleep, a characteristic which seems not to apply to any other component of the ascending arousal pathway.

Chapter 5: DO in the dopaminergic terminals of Nucleus Accumbens

5.1 Introduction

In chapter 4, we demonstrated that VTA DA neurons and more specifically their ability to produce DA in the VTA is necessary for the Meth-induced emergence of a 2ndC. It is well known that VTA DA neurons project to limbic and cortical areas via the mesolimbic and mesocortical pathway (Björklund & Dunnett, 2007) and one of the limbic areas that receives major VTA DA projections is the nucleus accumbens (NAc). It has been shown that extracellular DA in the NAc changes over the course of the sleep-wake cycle (Léna et al., 2005) but also fluctuates in synchrony with ultradian activity rhythms in the absence of the circadian oscillator (Blum et al., 2014). In addition, the NAc projections were reported to be one of the VTA projections that are able to promote arousal when optogenetically stimulated (Eban-Rothschild et al., 2016a). Extending on the evidence for signatures of rhythmicity in the NAc, our lab recently uncovered that intracellular calcium levels in the DA-neuronal terminals of the NAc also fluctuates in synchrony with ultradian locomotor activity rhythm albeit with an antiphasic relationship (Markam and Bourguignon et al, to be submitted). In contrast to observations in the NAc DA processes, calcium in the dopaminergic innervations of the dorsal striatum (DS) exhibited no rhythmic changes in concordance with ultradian activity rhythms (Fig. 5.1, adapted from (Markam and Bourguignon et al, to be submitted)). The antiphasic relationship of rhythms in cytosolic calcium versus locomotor rhythms is not unique to the NAc and has already been fiber-photometrically observed in the VIP neuronal population in the SCN, which are part of the SCN central circadian pacemaker (J. R. Jones et al., 2018). Therefore, given that DA

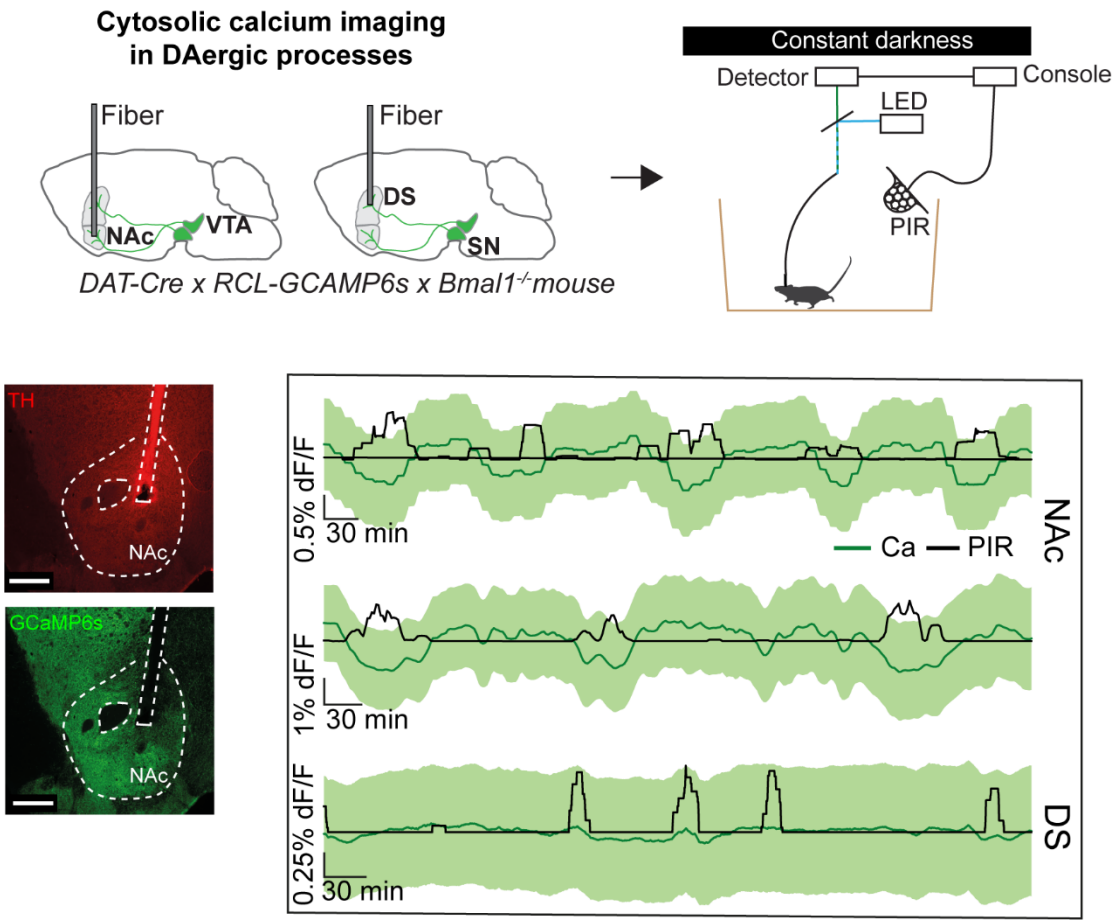


Figure 5.1: Rhythmic fluctuation of intracellular calcium in DA neuronal processes in the NAc

Top row, left: Experimental layout of optical fiber targeting DA neuronal terminals in the NAc that express the calcium indicator GCamp6 in Bmal1KO mice,
Top row right: simultaneous recording of locomotor activity by passive infra-red sensors clipped to the cage lid and calcium levels by fiber-photometric recording of calcium indicator fluorescence.
Bottom row, left: Confirmation of fiber placement in the NAc. Shown is a representative coronal section at the level of the NAc immunolabeled for GCaMP6 and tyrosine hydroxylase,
Bottom row, right: traces of indicator fluorescence (GCamp6,green), with standard deviation in light green, and locomotor activity(black trace) from NAc and dorsal striatum (DS). Adapted from Markam and Bourguignon et al, to be submitted

terminals in the NAc display oscillatory calcium signatures and that NAc/striatal dopamine can fluctuate in synchrony with locomotor activity, we hypothesized that NAc-projecting

DA neurons are crucial for the Meth-dependent emergence of a 2ndC and thus DO rhythmicity.

In the previous chapter, we demonstrated that ablation of VTA DA neurons or elimination of their ability to produce dopamine, leads to a loss of the capacity of 2ndC emergence in response to Meth. Here we intend to refine the DA neuronal population necessary for 2ndC/infradian rhythm production by employing a similar strategy of loss-of-function with the expectation that the ablated DA terminals at the central projection site of VTA DA neurons, the nucleus accumbens would also lead to loss of the 2ndC induction capacity. So far, in this thesis, we have demonstrated that the VTA circuitry is necessary for Meth-sensitive infradian rhythmicity. To determine if the NAc projecting VTA DA neurons are sufficient in driving these rhythms, we wish to employ a chemogenetic approach. It has been previously shown that in addition to Meth-treatment and DAT disruption, chemogenetic activation of midbrain DA neurons leads to period lengthening in circadian-deficient Bmal1KO mice (Blum et al., 2014). Thus, to observe significant period lengthening in activity rhythms, we are employing Bmal1KO mice for the chemogenetic manipulation.

To further delineate the DA cell population sufficient for period lengthening we aimed at delivering AAV that encodes a Cre-dependent chemogenetic activator (hM3dq) into the ventral population of the posterior VTA, as this VTA DA neuronal population seems to preferentially project to the NAc. Furthermore, we also wished to determine if this chemogenetically mediated period lengthening can be counteracted by antipsychotic treatment which has been already shown to rein in Meth-induced period lengthening in clock deficient Bmal1-KO mice (Blum et al. 2014).

5.2 Experimental design

To test whether NAc DA projections are necessary for 2ndC generation, we injected wildtype mice with 6-OHDA (2.5ug) in the NAc bilaterally to limit the ablation to dopaminergic terminals while the control groups were injected with the same volume of saline. Animals were then exposed to Meth to assess the functionality of the DO.

For chemogenetic activation of the VTA, we injected DAT-Cre Bmal1 KO animals with AAV-DIO-hM3Dq-mCherry, which encodes the Cre-activatable actuator hM3Dq into the ventral medial VTA in order to preferentially drive its expression in NAc projecting DA neurons. The Bmal1KO model was utilized for this study as period lengthening by chemogenetic means is more readily observable in circadian deficient than intact mice (unpublished data).

Animals with unilateral or lack of 6-OHDA ablation i.e., when TH immunostaining was observed in either or both hemisphere(s) in the nucleus accumbens region, were not included for the analysis. A few animals that didn't survive during Meth administration were also excluded.

5.3 Results

5.3.1 Selective targeting of DA neuronal projections in the NAc

The 6-OHDA-mediated ablation of DA innervations in the NAc was confirmed by the loss of TH-stained processes in the NAc. Compared to controls (Fig. 5.2a), immunolabeling for the DA neuronal marker TH was greatly reduced in the NAc of 6OHDA-injected

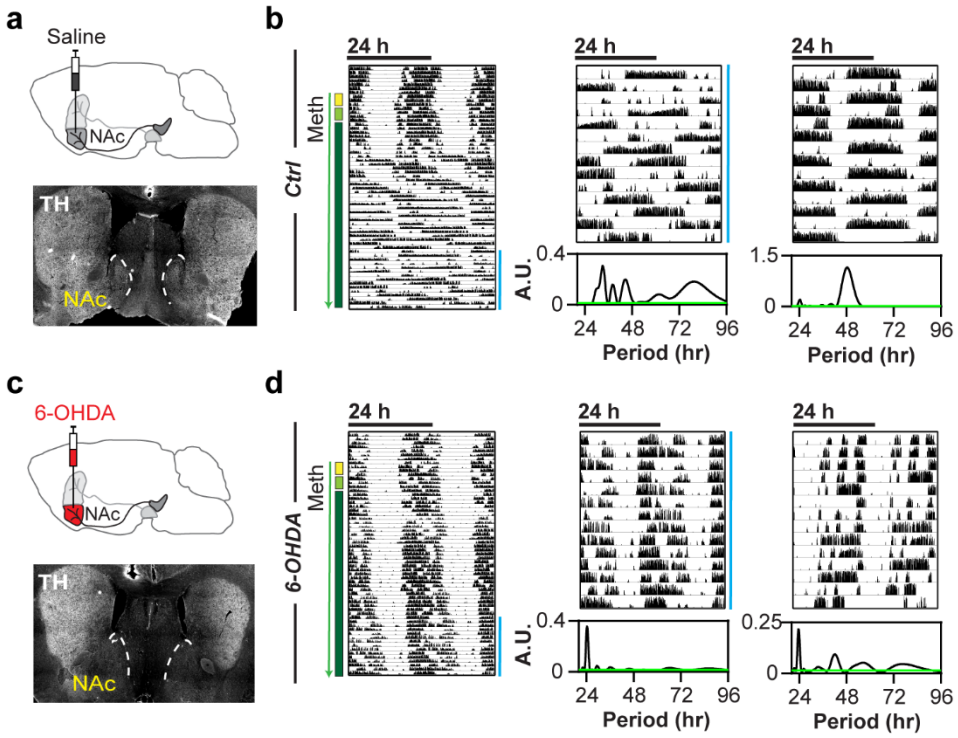


Figure 5.2: Meth-mediated infradian activity patterns are abolished upon ablation of NAc-projecting DA neurons

a,c) Schematics illustrating delivery of either saline or 6-OHDA in the VTA and representative coronal sections of the striatum immuno-labeled for TH. Absence of TH staining preferentially in the medial shell of the NAc indicates effective DA process elimination by 6-OHDA. **b,d)** Representative 60 days actograms on the left displaying running wheel activity patterns of control and 6-OHDA mice in constant darkness in response to escalating concentration of Meth; bar on the left indicates 25 (yellow), 50 (light green), 100 (dark green) mg/L Meth in drinking water. Right: shown are the final 14 days of the 60day recording (indicated by the blue bar and its Lomb-Scargle periodogram alongside an additional representative actogram from a different animal.

animals (Fig. 5.2c). We then assessed the effect of ablated ^{NAc}DA processes on spontaneous locomotor activity rhythms and Meth-mediated 2ndC induction.

5.3.2 Ablation of NAc DA terminals affects 2ndC emergence but not spontaneous locomotor rhythms

As shown by the representative actograms (Fig. 5.2b,d), ablation of NAc-projecting DA neurons led to the loss of Meth-mediated infradian rhythmicity (Fig. 5.2d), while control animals exhibited infradian activity patterns (Fig. 5.2b), as expected. The associated

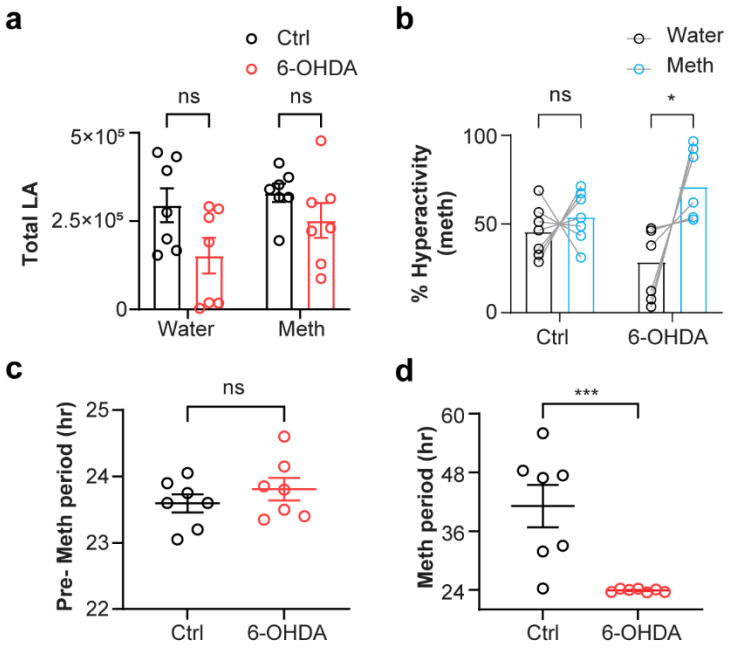


Figure 5.3: Preserved locomotor response but absence of infradian rhythm induction upon Meth exposure of 6-OHDA injected mice

a) Daily total locomotor activity (10-day averages) prior and during treatment with 100mg/L. Group data are means±SEM. Two-way ANOVA with Sidak's comparison test. ns, not significant. **b) %** Meth-induced hyperactivity displayed by 6-OHDA mice and its control. Compared are the normalized total locomotor activity (10 days averages) prior and during exposure to 100mg/L Meth. **c,d)** Periods of the highest periodogram peak prior to and during 100mg/L Meth exposure of 6-OHDA injected mice and controls (Crtl). Group data are means±SEM. Unpaired non-parametric t-test with Mann Whitney U test. ns, not significant, *P<0.05, ***P<0.001,

periodograms of ^{NAc}6-OHDA animals showed a lack of dominant infradian periodicities, while retaining a substantial PSD peak in the circadian range in the presence of a high concentration of Meth (100mg/L), further underscoring the loss of infradian drive and full preservation of circadian rhythmicity upon 6-OHDA treatment.

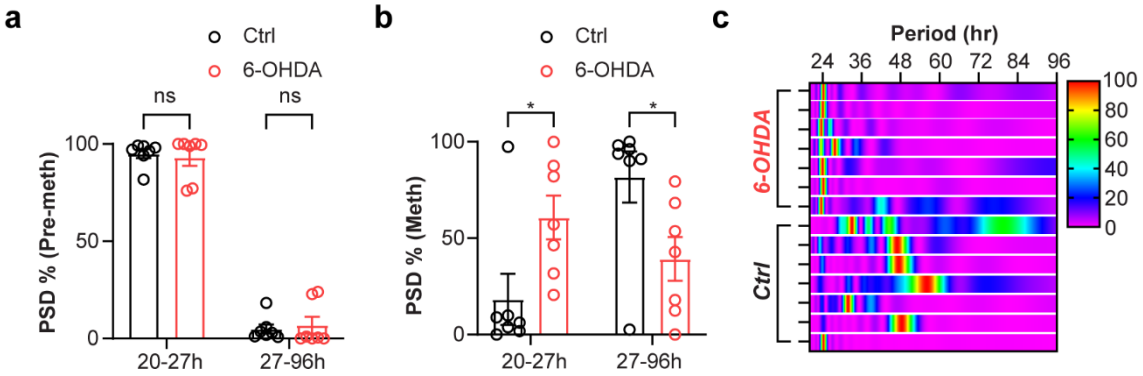


Figure 5.4: Profound loss of the capacity to produce infradian rhythmicity in mice upon ablation of NAc-projecting DA neurons

a) Distribution of PSD (%) of significant rhythmicities in the circadian 20-27hr and infradian 27-96hr range prior and **b)** during 100mg/ml Meth exposure. Group data are means±SEM. Two-way ANOVA with Sidak's comparison test. ns, not significant, *P<0.05. **c)** Composite heatmap displaying periodograms of individual animals derived from 14 days of recoding during Meth

The free running periodicity of the animals prior to and during Meth treatment was similarly to Casp3 and THKO mice, not affected by the manipulation. 6-OHDA-injected mice displayed robust circadian periodicity prior to Meth and remained circadian throughout the Meth treatment (Fig. 5.3c,d). As expected and in contrast to ablated animals, Meth-treatment shifted the dominant period into the infradian range in control animals (Fig. 5.3d). These results underscore that 6-OHDA-mediated ablation of the NAc projections leads to a loss of the infradian rhythm generation capacity. Notably, unlike Casp3 and THKO mice, 6-OHDA injected animals did not exhibit a significant decrease in total LA

when compared to controls under both exposure to regular water only or during Meth treatment (Fig. 5.3a). Yet, ablated animals showed a hyperlocomotor response to Meth (Fig. 5.3b) further corroborating that elimination of the NAc projecting neurons leads to the loss of infradian rhythm generation but not the classical psychostimulant response, which is hyperlocomotion.

To further assess the loss of infradian rhythm emergence upon 6-OHDA injection, we calculated the PSD distribution between circadian and infradian periodicities in the presence and absence of Meth. As expected, there was no difference between control and experimental animals prior to Meth. In both groups, rhythmic power was largely confined to the 20-27hr period range (Fig. 5.4a).However, while the infradian contribution increased in 6-OHDA group, it was still significantly lower than in the control group in the presence of Meth (Fig. 5.4b). This shift in infradian rhythmicity which was not found in Casp3 and THKO can be attributed to multiple rather low amplitude periodicities in the very near infradian range as can be deduced from the heatmap-display of the periodograms of the 6-OHDA group (Fig. 5.4c). While robust, this PSD% metric is not a reliable measure if the circadian periodogram peak is not very pronounced and the infradian range at the same time shows low amplitude peaks which could result from intra alpha band (= wake bout) variability due to e.g., estrus cyclicity. Under such circumstance, AUC analysis will falsely report DO mediated infradian activity, while the actogram inspection would not support such conclusion due to the lack of drifting activity bouts. Therefore, the highest peak analysis is a helpful complementary approach to assess infradian rhythmicity emergence alongside visual inspection of periodograms. In case of the 6-OHDA experiment, the highest peak determination (Fig. 5.3d) confirms the absence

of significant infradian periodicities in the 6-OHDA animals suggesting that DA neuronal innervations to the NAc are key for infradian rhythm generation.

5.3.3 NAc projecting DA neurons drive period lengthening which is counteracted by antipsychotic treatment

We employed the clock-deficient ultradian model of rest activity, Bmal1KO mice which additionally harbored the DAT-Cre transgene to limit expression of the chemogenetic actuator to the dopamine neurons (Fig.5.5a). The animals were injected with an AAV encoding the Cre-activatable h3Mdq into the ventro-posterior VTA. The animals were then exposed to the h3Mdq-ligand CNO via drinking water to activate the actuator-

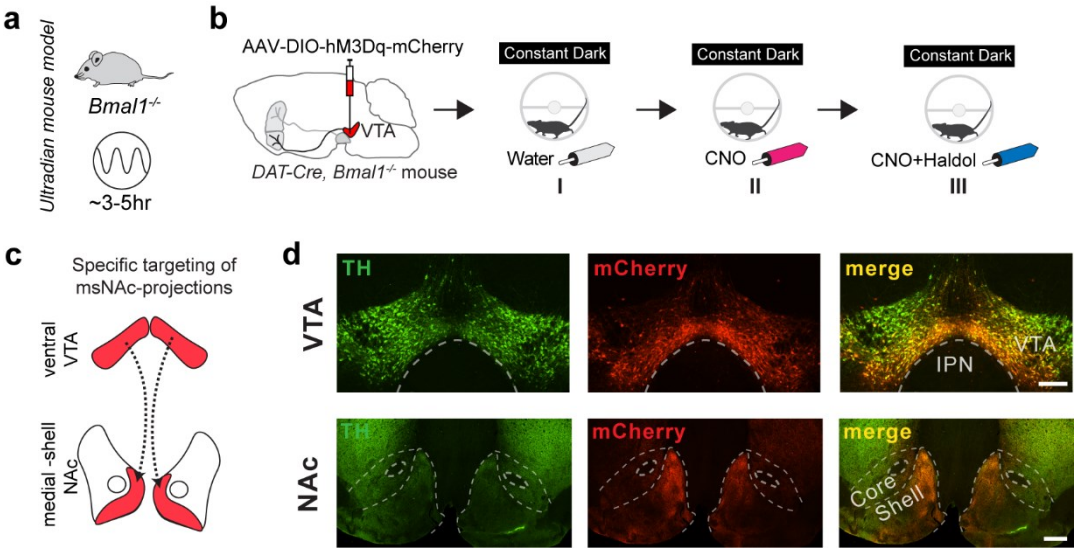


Figure 5.5: Expression of the chemogenetic actuator mCherry-tagged h3Mdq in DA neurons of the medial VTA that project to the medial shell of the NAc

a) Clock-deficient Bmal1KO mice producing ultradian activity rhythms.
b) Illustration of experimental timeline: DAT-Cre x Bmal1KO mice were first injected with injected with Cre-activatable chemogenetic actuator hM3Dq in the VTA, followed by activity monitoring under drug treatment. **c)** Schematic indicating the projections of DA neurons of the ventro-medial VTA to the medial shell of the NAc.
d) Representative coronal sections of NAc and VTA stained with TH and mCherry demonstrate preferential infection of DA neurons in the ventro-medial VTA that project predominantly to the medial shell region of NAc.

expressing DA neurons. After 7 days of CNO treatment, the antipsychotic haloperidol, which has been previously shown to shorten Meth-induced period lengthening in BmalKO mice (Blum et al. 2014) was added to the CNO-containing drinking water to assess its affect on CNO induced period lengthening (Fig 5.5b). The expression of the mCherry-

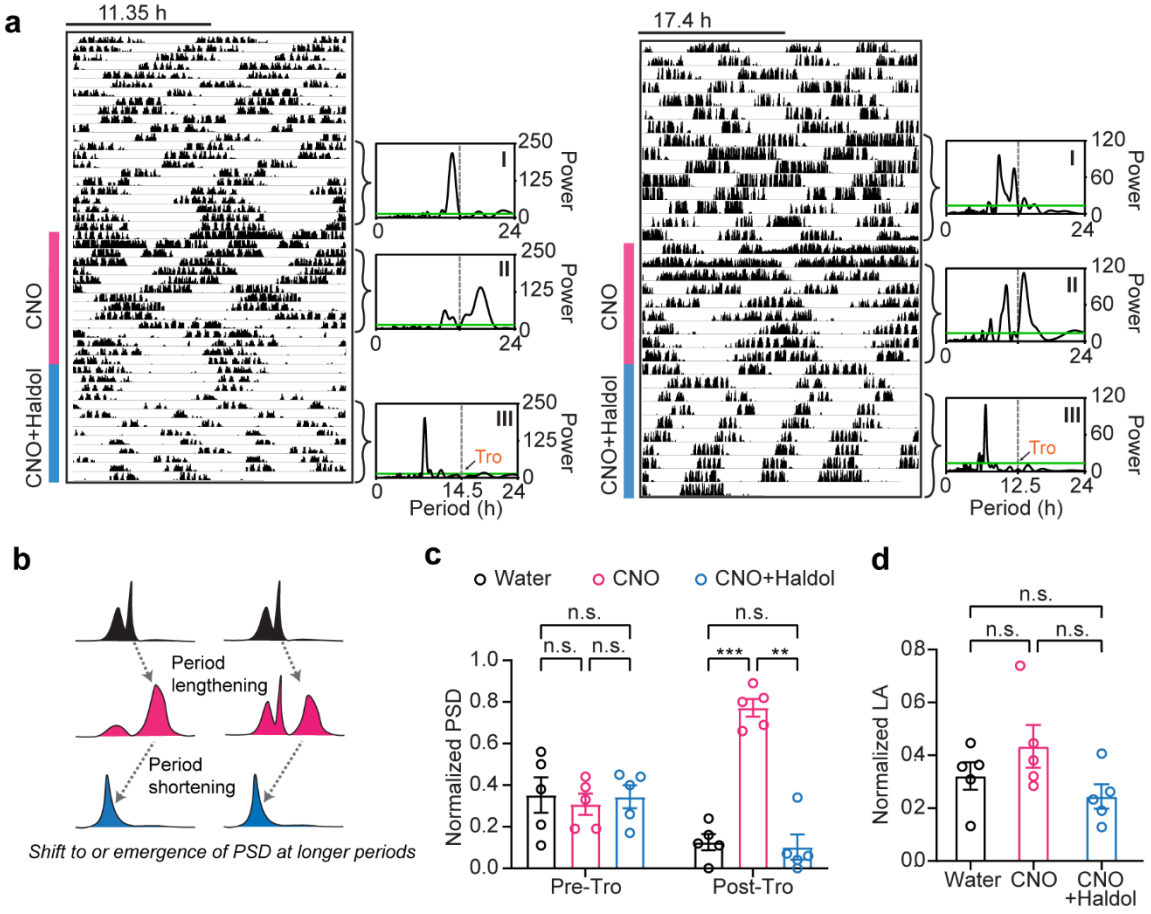


Figure 5.6: Chemogenetic activation of VTA-NAc circuitry is sufficient to lengthen ultradian period in Bmal1Ko mice

a) Representative, modulo actograms displaying running wheel activity patterns in response to CNO in drinking water followed by CNO+Haldol. 4 days Periodgrams displaying the oscillations from the indicated time window. Grey dotted line marks trough (Tro) after the dominant peak prior to CNO treatment ('Water', I).
b) Illustrations of the shift in the PSD in response to drug treatments.
c) Normalized PSD of short periodicities ranging from 0-Tro (Pre-Tro) and Tro-24hr (Post-Tro) during I: Water, II: CNO, III: CNO+Haldol . **d)** Normalized total locomotor activity for 4 days in I, II and III. Group data are means±SEM. Two-way ANOVA with Bonferroni's multiple comparisons. ns, not significant, **P<0.01, ***P<0.001.

tagged DREADD hM3Dq was subsequently assesses by immunostaining with mCherry and TH antibodies. At the injection site, hM3Dq expression was found largely limited to the dopamine neurons of the ventro-medial VTA and immunolabeling across the NAc region demonstrated profound enrichment of mCherry fluorescence in DA projections in the medial shell region (Fig. 5.5c).

Addition of CNO to the drinking water of virus-injected animals resulted in period lengthening of the locomotor activity rhythms and as shown by the shift of ultradian oscillations in the periodograms towards the circadian range (Fig. 5.6a). To assess the extent of period lengthening, similar to the Meth PSD analysis, we defined the trough (Tro) of the first peak in the periodogram prior to CNO. Any manipulations that lead to period lengthening would shift the peak PSD post-Tro i.e., towards the circadian range as illustrated (Fig. 5.6b). We found that CNO resulted in a significant shift in the PSD Post-Tro while Haldol treatment along with CNO, reversed the CNO driven period lengthening. We also found that neither treatment, CNO or CNO+Haldol significantly altered the total locomotor activity. These results indicate that the NAc projecting DA neurons of the VTA are sufficient to enable lengthening ultradian rhythms in Bmal1KO mice and that haloperidol is able to reverse this DREADD mediated lengthening as is the case with Meth-mediated period lengthening.

5.4 Conclusion

The mesolimbic VTA-NAc pathway is well known to play a central role in reward-biology (Russo & Nestler, 2013) but has also been implicated in regulating sleep-wake related behaviours (Sulaman et al., 2023). Here we provide evidence that the NAc projecting VTA DA neurons may serve as elements of the DO or even harbor it.

We show that upon ablation of the DA neuronal innervations to the NAc, the animal's ability to produce a Meth-induced 2ndC is largely lost. We also show that this manipulation if anything only mildly affects the overall locomotor activity, which is in contrast to the more severe hypolocomotor phenotype upon Casp3-mediated, DA neuron ablation across the VTA.

Chemogenetic activation of midbrain DA neurons have been previously shown to lengthen the locomotor activity period in clock-deficient mice (Blum et al., 2014). Here we provide evidence that chemogenetic activation of NAc projecting VTA DA neurons in particular is sufficient to lengthen ultradian locomotor activity rhythms. We further demonstrate that the activating DREADD we employed here is likely acting on the same DO oscillatory process as Meth does as in both cases the antipsychotics Haldol is able to counteract the induced period lengthening. Notably, the dopamine 2 receptor (DRD2) is the main target of Haldol, where it acts as an inverse agonist. As the DRD2 is also expressed presynaptically on DA neurons it seems conceivable that Haldol may exert its action on the DO via direct action on VTA DA neurons, thereby possibly affecting TH activity (Bello et al., 2011). In conjunction with the findings that extracellular DA in the striatal/NAc region and calcium levels in the NAc DA terminals were both found to fluctuate in synchrony with locomotor rhythms (Blum et al., 2014; Markam and Bourguignon et al, to be submitted), our data on NAc projecting DA neurons presented in this chapter strongly supports the notion that these neurons harbor the DA oscillator as it seems that they are sufficient to lengthen non-circadian locomotor rhythms and necessary for the production of infradian rhythms.

Chapter 6: Mode of DA release in the context of DO action

6.1 Introduction

In chapter 4, we demonstrated that DA biosynthesis in DA neurons of the VTA is necessary for infradian rhythm emergence and by extension for the dopaminergic oscillator to function. Upon synthesis, DA is packaged into vesicles via the vesicular monoamine transporter 2 (Vmat2) and thereby maintaining a reserve pool of DA until the DA terminals are depolarized leading to vesicular fusion with the cell membrane to release DA into the extracellular space (Eiden et al., 2004; Liu & Kaeser, 2019). When amphetamines enter the dopaminergic terminals via DAT or by lipophilic diffusion through the plasma membrane, it sets the Vmat2 transporter in reverse which in turn leads to the DA exit from the vesicles thereby increasing intracellular DA levels (Nickell et al., 2014; Sulzer et al., 2005a). Interestingly midbrain slice culture studies from VmatKO mice showed that amphetamine can still induce DA release from DA neurons in the absence of Vmat2, however the amount of DA release is significantly reduced (Fon et al., 1997). On the other hand, when DAT is disrupted, there is no DA efflux upon amphetamine administration, suggesting that DAT is essential for amphetamine based DA release (S. R. Jones et al., 1998). However, DAT disruption can also lead to 2ndC emergence similar to Meth (or amphetamine) (Fig. 6.1). Both manipulations result in an increase in extracellular DA (S. R. Jones et al., 1998; Seiden et al., 1993) which may be critical for the ensuing period lengthening of the DO and the emergence of a 2ndC (Blum et al., 2014). While Meth is able to reverse DAT mediated transport causing DA efflux, DAT disruption inhibits DA uptake thereby increasing DA levels. In the latter case it is not clear though how DA enters the extracellular space, it is possible that DA release via Vmat2

expressing vesicles is of critical importance in DAT disrupted mice to generate infradian rhythms. Together, these findings show that both vesicular (DAT KO) and DAT mediated (Meth) increase in extracellular DA can result in the emergence of the 2ndC.

Since the amount of extracellular DA influences the emergence and periodicity, this chapter is aimed at determining if the disruption of Vmat2 which causes reduction in Meth associated DA release (Fon et al., 1997), affects the periodicity of Meth-associated infradian rhythms. As 2ndC is manifested in a state of hyperdopaminergia, we also wish to demonstrate that the specific mode of DA release is irrelevant as long as it can facilitate the required hyperdopaminergic state.

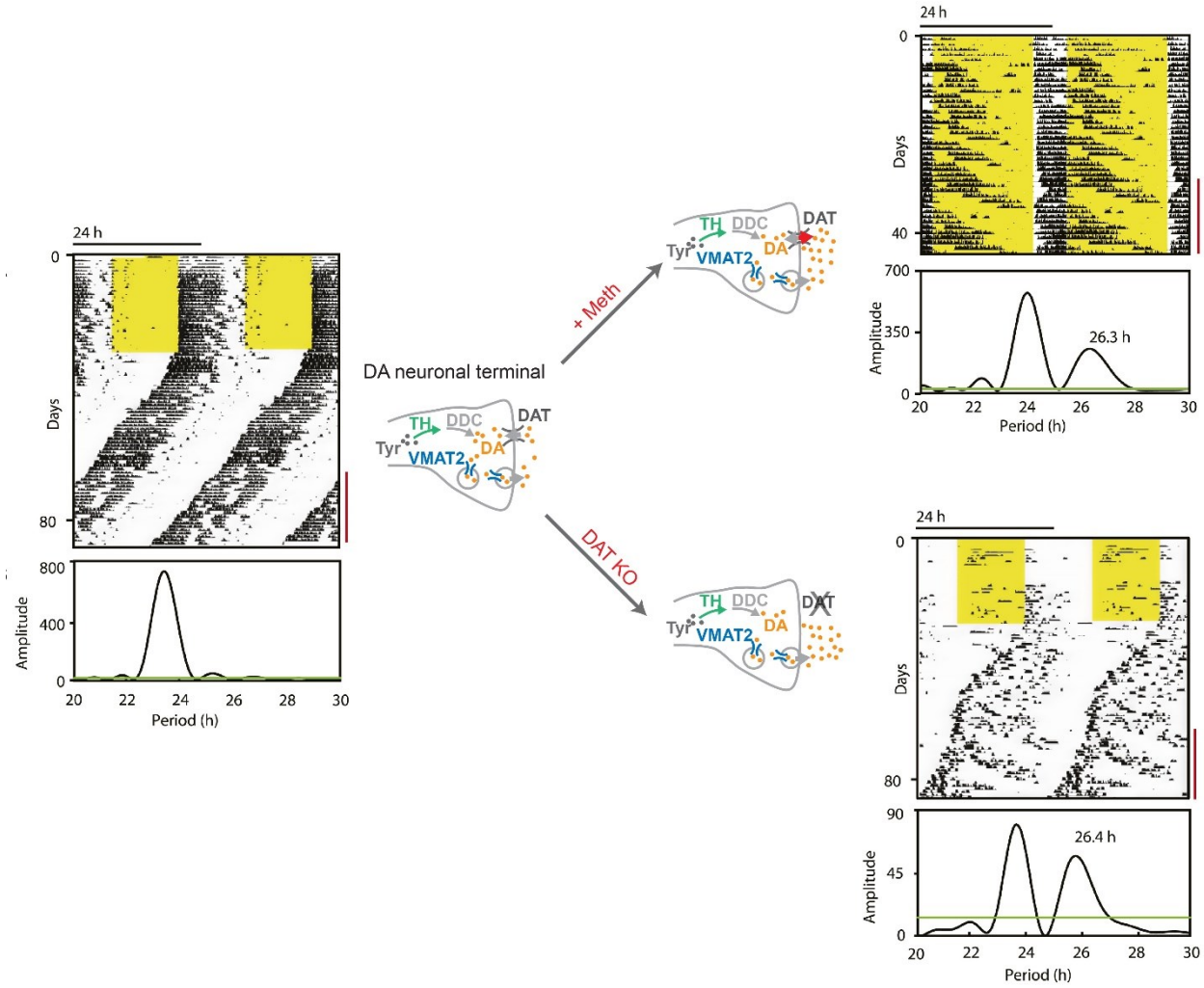


Figure 6.1: 2ndC emergence in response to both DAT transport reversal (Meth) and DAT elimination (DATKO)

Left: Actogram of a control mouse displaying circadian running wheel activity.
Middle: The schematic illustrates the DA synthesis and release pathways in DA neuronal terminals of Meth-treated and DATKO mice, respectively.
Right, top: Actogram of a Meth exposed animal displaying infradian rest:activity pattern and a 2ndC with a 26.3hr periodicity as indicated by the periodogram underneath the actogram. The respective schematic illustrates the reversal of DAT by Meth leading to an increase in extracellular DA. **Right, bottom:** Actogram of a DATKO animal displaying infradian rest:activity pattern and a 2ndC with a 26.4hr period; the associated schematic shows the inhibition of DAT leading to an increase in extracellular DA. Adapted from Blum et al., 2014

6.2 Experimental design

To further understand the importance of the mode of DA release by DA neurons of the VTA in the context of infradian rhythm generation, we targeted the Vmat2 mediated packaging of DA into synaptic vesicles. To disrupt Vmat2, we injected AAV-GFP-Cre bilaterally into the VTA of $Vmat2^{fl/fl}$ mice (Fig. 6.2a). The consequences on infradian rhythmicity was then assayed by monitoring running wheel activity upon gradual introduction of Meth into the drinking water up to a concentration of 100mg/L.

Animals with partial Vmat2 disruption in the VTA were not included for the analysis. A few animals that didn't survive during the Meth administration were also excluded.

6.3 Results

6.3.1 Vmat2 is not required for Meth-mediated DO rhythm generation

Loss of Vmat2 expression in $Vmat2^{fl/fl}$ mice injected with AAV-GFP-Cre was confirmed by in-situ hybridization. A selective absence of Vmat2 riboprobe signal was observed in the VTA of Cre virus injected animals (Fig. 6.2b). Only mice that showed a complete loss of Vmat2 message in the posterior VTA were considered for analysis. We also inspected the expression of Vmat2 in the neighboring raphe nucleus and the locus coeruleus confirming that elimination of Vmat2 was limited to the midbrain VTA area (Suppl. Fig. 6.1).

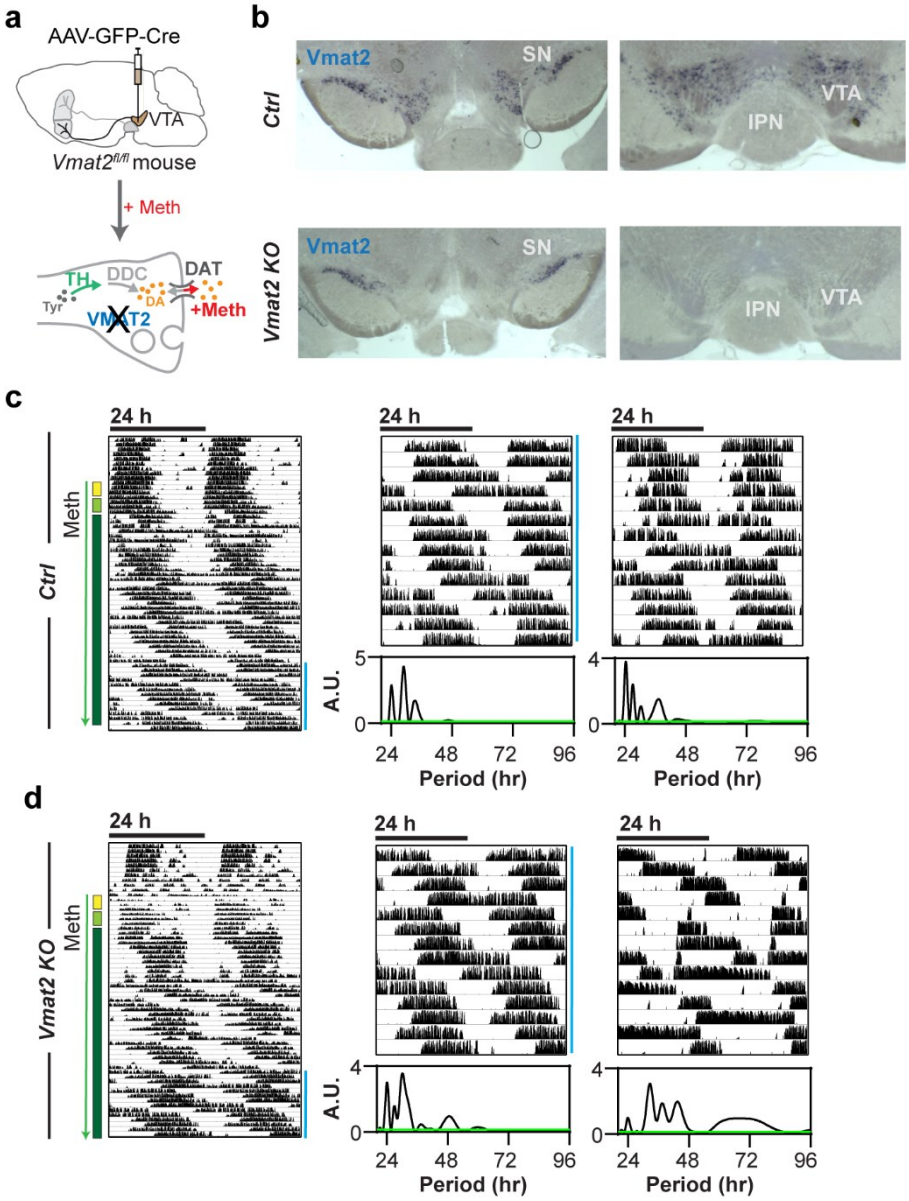


Figure 6.2: Infradian rhythm induction upon Vmat2 disruption in the VTA

a) Cartoon illustrating the injection of AAV-GFP-Cre in the VTA of *Vmat2*$^{fl/fl}$ mice resulting in the expected loss of vesicular DA release. **b)** Representative coronal sections from the SN and VTA after in situ hybridization with a Vmat2 riboprobe. Lower right image confirms Vmat2 loss across the VTA upon injection of the Cre virus while the SN is spared as shown in the lower left. **c,d)** Representative 60 days actograms on the left displaying running wheel activity patterns of control and Vmat2KO mice in constant darkness in response to escalating concentrations of Meth (25, yellow; 50, light green; 100mg/L, dark green) in drinking water. Right: replot of the final 14 days from the 60 days actogram indicated by the blue bar and corresponding Lomb-Scargle periodogram alongside the actogram of a second representative animal .

6.3.2 Vmat2 disruption in the VTA doesn't affect the infradian rhythm generation

We then assessed whether ^{VTA}Vmat2KO animals are capable of generating infradian rest-activity patterns in response to Meth. Both, control and ^{VTA}Vmat2KO animals exhibited infradian locomotor rhythmicity under Meth exposure (Fig. 6.2c,d). This is also evident in the associated periodogram as they exhibit multiple peaks in the infradian 27-96hr range in response to Meth (Fig. 6.2d, shown below the actograms).

Disruption of *Vmat2* in the VTA resulted in a profound reduction of spontaneous locomotion (Figs. 6.2d and 6.3a) similar to or even stronger than what has been observed

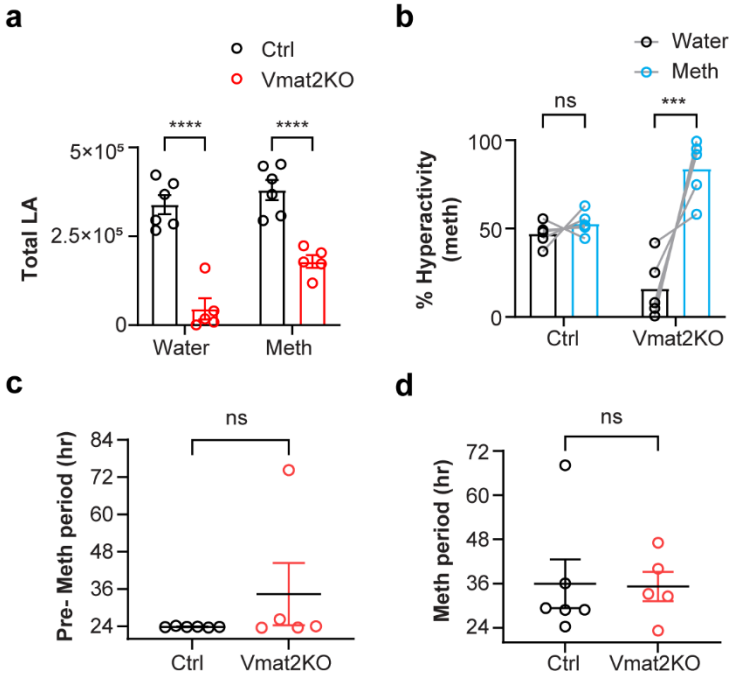


Figure 6.3 : Meth induced activity in the absence of DA in the VTA

a) Daily locomotor activity averages from 10 consecutive days prior or during 100mg/L Meth of Vmat2KO mice and controls. **b) %** Meth-induced hyperactivity displayed by Vmat2KO mice and their controls. Group data are means±SEM. Two-way ANOVA with Sidak's comparison test. ns, not significant , ***P<0.001, ****P<0.0001. **c,d)** Period of the highest periodogram peak prior to and during 100mg/L Meth treatment. Group data are means±SEM. Unpaired non-parametric t-test with Mann Whitney U test. ns, not significant.

in the ^{VTA}THKO and ^{VTA}Casp3 mice as reported above. Vmat2KO mice however showed strong hyperlocomotion in response to Meth, seemingly surpassing the locomotor levels of control animals, which did not exhibit much change in response to Meth (Fig. 6.3b).

We then identified the dominant period of Vmat2KO animals prior to and during Meth, by determining the period position of the dominant peak in the respective periodograms. In almost all animals in both the controls and the Vmat2KO group, locomotor activity was dominated by circadian periodicities prior to Meth (Fig. 6.3c). Upon Meth exposure, the dominant period moved into the infradian range for both Vmat2KO and control animals to a similar extend and there was no difference in their periodicities (Fig. 6.3d). Thus, the capacity to generate a 2ndC was retained in Vmat2 KO mice despite a tendency to extremely low spontaneous locomotor activity levels when exposed to water only, i.e., prior to Meth treatment. This was further supported by the shift of PSD from the circadian range dominated state prior to Meth treatment (Fig.6.4a) to a state prone of infradian power (Fig. 6.4b). The extend of this shift was indifferent between Vmat2KO mice and controls underscoring an unimpaired capacity for 2ndC induction upon Vmat2 removal from the VTA. The composite heatmap display of individual periodograms computed from locomotor recordings confirms this: during Meth treatment, Vmat2KO animals show infradian range oscillations which appear slightly more pronounced than in the control animals (Fig. 6.4c). Indeed, preliminary data (not shown) suggests that Vmat2 removal results in an earlier onset of 2ndC emergence and a faster period increase towards the maximal period reached, which all together point to an actual facilitation of 2ndC generation through Vmat2 disruption.

Together these results demonstrate that Vmat2 disruption in the VTA doesn't impair the infradian generation capacity of mice suggesting that vesicular DA release is dispensable for the manifestation of 2ndC during Meth treatment.

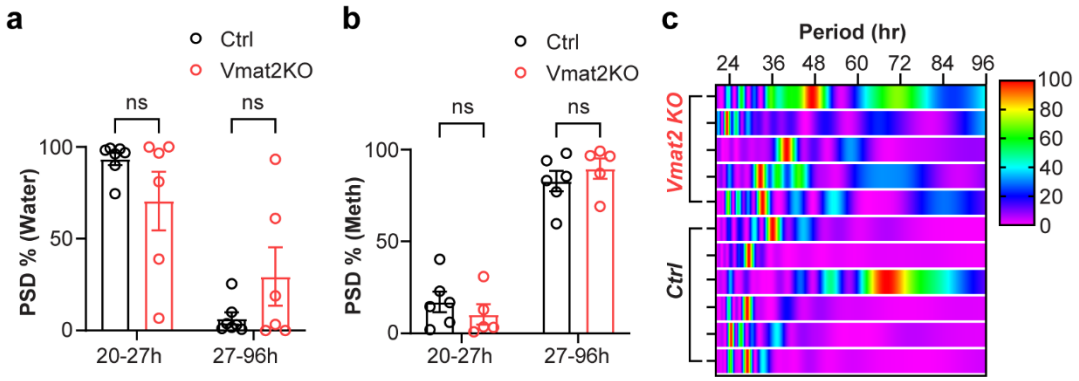


Figure 6.4 : Infradian generation capacity remains unaffected in ^{VTA}Vmat2KO mice

a) Distribution of PSD (in %) of significant rhythmicities in Vmat2KO and its control across the circadian 20-27hr and infradian 27-96hr range prior to Meth treatment and **b)** during 100mg/L Meth. Group data are means±SEM. Two-way ANOVA with Sidak's comparison test. ns, not significant. **c)** Composite heatmap displaying normalized individual periodograms computed from 10day of consecutive activity data during Meth treatment demonstrating infradian periodicities in both Vmat2KO mice and controls during Meth treatment.

6.4 Conclusion

Vmat2 is critical for vesicle mediated synaptic release of DA, however it seems not necessary for Methamphetamine mediated release of DA (Fon et al., 1997), as this likely relies exclusively on DAT transport reversal (S. R. Jones et al., 1998). We here extend this finding and show that Vmat2 in the VTA is also not necessary for Meth associated infradian rhythm generation originating in the VTA-NAc circuitry.

We demonstrated this by selective disruption of Vmat2 expression across the VTA upon which the animal's ability to generate 2ndC in the presence of Meth was retained. However, this may not be the case for 2ndC emergence that is elicited by DAT disruption

under the assumption that high extracellular DA levels cannot be attained without Vmat2 action and corresponding vesicular DA release. Given that there is no evidence for DA to be able to exit the DA cell by means other than vesicular release or DAT transport reversal it seems conceivable that disruption of the Vmat2 gene in the VTA of DATKO mouse would abolish its capacity for 2ndC induction as DA cannot exit the DA neuron.

We also found ^{VTA}Vmat2KO animals to exhibit severe hypolocomotion in the absence of Meth similar to ^{VTA}Casp3 and ^{VTA}THKO mice yet were equally potent as their control littermates in producing a 2ndC. This finding therefore argues against the view that the lack of the 2ndC induction capacity in ^{VTA}Casp3 and ^{VTA}THKO mice is due to a global and severe impact on locomotion or even general animal well-being precluding the animals from undergoing profound changes in rhythmic arousal.

The fact that neither elimination of DA vesicular release nor DA release by the DAT does abolish 2ndC emergence indicates that while DA needs to be released by DA neurons of the VTA for infradian rhythm production, the mode of release is not of concern. This finding also suggests that DAT or the vesicular packaging and release machinery do not represent elements that are specific for the DO rhythm generation process, i.e., do not represent core components of this oscillator.

The view that Meth reverses DAT action readily but Vmat2 transport only at high concentrations (Seiden et al., 1993) may explain why Meth may in fact induce a 2ndC more readily in Vmat2KO animals. In intact animals, the maximum efflux of intracellular DA might only take place at high Meth concentrations which also lead to the extraction of DA from all DA containing vesicles. As a consequence, 2ndC induction may require exposure to high concentration of Meth for a considerable time. In Vmat2KO mice

however, all intracellular DA is cytoplasmic and thus it is conceivable that mere DAT reversal which already takes place at relatively low Meth concentrations produces high levels of extracellular DA. As consequence, 2ndC is induced more readily.

While the role of Vmat2 in 2ndC induction and periodicity of DO rhythms needs further investigation, it is clear that Meth-mediated rhythm generation does not require vesicular DA release, however since DAT disruption and chemogenetic lengthening of rhythms must rely on vesicular DA release, Vmat2 might be critical for Meth-independent DO rhythm generation.

To summarize, we conclude that the mode of DA release from the VTA-NAc circuitry is not of relevance for the ability to induce a 2ndC as long as the outcome is elevation in extracellular DA levels.

Chapter 7: Discussion

7.1 Summary of findings

In this body of work, we advance our understanding of the properties, neural locus, and the underlying mechanisms of a secondary, dopamine-based rest-activity driving oscillator, the DO. We showed that the DO can attain a wider range of periodicities even in the presence of the circadian oscillator, with animals exhibiting periods from 26hrs to 72 hrs. We employed this unique feature of oscillator action, the emergence of 2ndC under Meth treatment as a means to test DO functionality. We demonstrate that selective ablation of the DA neurons in the VTA or disruption of DA biosynthesis in the VTA leads to the loss of the animal's ability to exhibit 2ndC under Meth treatment. Furthermore, we show that emergence of this 2ndC is also impaired upon ablation of the DA innervations in the NAc, the major projection site of VTA DA neurons. This targeted approach at the VTA-NAc DA circuitry thus reveals its critical role in driving DO rhythms. We strengthen this argument by showing that chronic chemogenetic mediated activation of NAc-projecting VTA neurons is sufficient to lengthen the DO rhythms and that this lengthening is counteracted by antipsychotic treatment similar to Meth-mediate period lengthening underscoring that chemogenetic activation of VTA neurons and Meth treatment affects the very same oscillator, the DO. Finally, we show that disruption of Vmat2, the vesicular transporter required for dopamine packaging and consequently its synaptic release is not necessary for Meth mediated emergence of the 2ndC. This finding together with previous data on DAT-disruption mediated 2ndC mergence (Blum et al., 2014), indicates that the mode of DA release in DO rhythm generation is not relevant as long as DA can exit the VTA DA neuron.

Since DA is inherently linked to locomotor activation and action of Methamphetamine (Sulzer et al., 2005b; Wisor et al., 2001), it can be argued that the loss of 2ndC emergence is rather a result of the hypoactivity we observed upon VTA DA neuron manipulation or simply the loss of the site of Meth action. Here we demonstrate that 2ndC emergence is abolished irrespective of whether the locomotor activity is reduced as in case of the VTA Casp3 and THKO models or not, as observed in NAc 6-OHDA mice. In addition, we also demonstrate that Meth-induced hyperactivity is preserved even in the absence of DA neurons or its production capacity in the VTA.

Together, these results indicate that the DO is a highly tunable oscillator that is likely located within the midbrain VTA dopaminergic population that project to the NAc and while the biosynthesis of DA in the VTA is required for rhythm generation, the mode of its release is not critical for its functionality. In addition, the differential effects of Meth in the absence of VTA-NAc DA circuitry revealed that hyperlocomotion induction and period lengthening of locomotor rhythms are two separable effects of chronic Meth exposure via drinking water.

7.2 General discussion

Here we provide a detailed interpretation of the finding presented and limitations of the study on the dopamine-based oscillator, DO or otherwise known as MASCO (Methamphetamine sensitive circadian oscillator).

7.2.1 Tunability of the DO

We began by first challenging the view that DO/MASCO driven, Meth-dependent rest-activity rhythms have a limited periodic range in the presence of a functioning circadian

clock . We have previously shown that this dopaminergic oscillator drives a 2nd rhythmic locomotor component (2ndC) which can regularly adopt a wide range of periodicities from ultradian to far-infradian range, however, this was only reported for animals in the absence of a functioning circadian clock. Notably, the tunability of the oscillator was uncovered upon application of escalating concentrations of Meth in the drinking water, arguing for a direct dependency of the oscillator period on Meth concentration (Blum et al., 2014). Despite displaying such a wide range tunability in the special case of clock deficient mice, the DO/MASCO maximal periodicities in the presence of an intact the circadian clock have been previously reported to be largely limited, typically only reaching 26-30hr, in line with the view that the DO/MASCO represents a second circadian oscillator located outside the SCN (Tataroglu et al., 2006; Taufique et al., 2022). Therefore, by means of Meth, we tested the tunability of the DO and demonstrated that this second oscillator can drive sleep-wake rhythms to 72 hrs in the presence of the circadian oscillator. In fact, we found that at 100mg/L Meth in the drinking water, over time at least a third of the animals will adopt periodicities of 48hrs and beyond (see Fig. 3.5b) suggesting that far infradian periodicities are not a rare occasion but a rather typical outcome when the DO is activated enough by Meth. We believe that the periodicity of this oscillator can be potentially lengthened further if provided with Meth at a concentration higher than 100mg/L, however our objective was to demonstrate that the DO/MASCO can readily acquire periodicities far beyond the circadian range. The DO was formerly referred to as a dopaminergic ultradian oscillator (DUO) by our group, however we have dropped the attribute ultradian in this thesis due to the wide range of periodicities it can adopt. Given that high levels of Meth in the drinking water can routinely drive the 2ndC

into the far infradian range, the alternative descriptor, MASCO, seems equally inadequate and perhaps MASO, Methamphetamine sensitive oscillator lacking the circadian attribute, would be a more fitting term. Considering that the 2ndC that is observed upon DAT gene disruption (Blum et al., 2014) is sensitive to period shortening by the antipsychotic haloperidol as is the Meth-induced 2ndC and given that Meth increases extracellular DA by targeting the DAT as does DAT elimination, it seems very conceivable that both 2ndCs, the Meth-driven and the ones that results from DAT disruption, are the output of one and the oscillator process. As the DAT-disruption-induced 2ndC does not require Meth, we suggest to use the term DO for dopaminergic oscillator or dopamine-dependent oscillator when describing this secondary rest:activity rhythm generator, as it seems that dopamine is of core importance for its functionality but not Meth.

While a drinking water concentration of 100mg/L was able to drive 2ndCs into the far-infradian range with periods of 48+hrs, the actual period reached at any time during the exposure to this high Meth levels was quite variable within a given animal cohort. Notably, a large fraction of mice (~60-70%) never reached far infradian periodicities of 48hr and beyond but instead exhibited 2ndC with maximum periods between 28 and 48hrs. We also observed variability in the onset of infradian rhythmicity. While all mice used employed were of the same C57BL/6 background, it is possible that some genetic variability has been introduced due to spontaneous mutation and genetic drifting (Dumont, 2019) of the various mouse lines used to generate the compound genetic models described here. Such genetic variability and/or epigenetic variability can possibly account for the observed variability in the onset of 2ndCs and the actual period they may adopt (Taufique et al., 2022). While we have not observed sex differences in 2ndC

periodicities and thus used female and male mice indiscriminately, it is possible that sex could account for some variability in the 2ndC onsets. Preliminary data suggests that the other contributing factor could be the rapid introduction of Meth at high concentration (100mg/L). Since we aimed to demonstrate that the DO exhibits a wide tuning range even in circadian intact mice, we exposed the animals only briefly to 25mg/L and then 50mg/L before treating them with 100mg/L Meth to facilitate lengthening of 2ndC into the far-infradian range. As Meth can promote the internalization of DAT (German et al., 2012; Saunders et al., 2000) resulting in the inhibition of DA uptake, internalization will also cause a decrease of the Meth-mediated DA efflux via DAT. While these findings derive from acute injections of Meth, it seems conceivable that chronic exposure to high levels of Meth via drinking water may equally result in DAT internalization and thus desensitization to Meth, the degree of which possible depending on (epi)genetic/sex difference between individual members of a given experimental cohort, which in turn would translate in the observed 2ndC onset and period variabilities. The maximum period reachable and the time course of its manifestation might thus be a function of the time course of Meth dose escalation in conjunction of (epi)genetic and sex differences. In this context, assessment of DAT internalization might by a meaningful starting point towards understanding 2ndC onset and period variability moving forward.

7.2.2 Location of the DO

The previous findings that DAT disruption led to period lengthening of ultradian rhythms in circadian clock deficient mice in constant darkness and emergence of a 2ndC in mice with a functioning circadian clock suggested that a canonical clock-independent oscillator must exists in a location within the dopamine system. The observation that chemogenetic

activation of DAT-expressing neurons in the midbrain is sufficient for period lengthening of this DO provides additional support for DAT-expressing DA neurons in the midbrain as site of the DO (Blum et al., 2014). This view further suggest that these DA neurons exhibit characteristics compatible with a rhythmic drive of sleep and wake. There are two major DAT expressing dopamine populations in the midbrain, the DA neurons of the SN and those residing in the VTA, both of which have already been implicated in the sleep-wake process. The SN DA neurons exhibit neural activity during the wake state and the VTA DA neurons fire during both the wake and the REM state of sleep (Brown et al., 2012). It has also been previously shown that NAc projecting VTA DA neurons are capable of maintaining wakefulness even during states of high sleep pressure, which in mice are associated with the first hours after lights on during the daily LD cycle (Eban-Rothschild et al., 2016a). In critical support of mesolimbic dopamine as site of the DO oscillator, the lab recently uncovered that DAT-expressing neuronal processes in the NAc of clock-deficient mice in constant darkness exhibit rhythmic fluctuation in intracellular calcium in synchrony with ultradian locomotor activity. The observations were made using NAc directed fiber photometry in mice where expression of the calcium indicator GCaMP6 was directed to DAT expressing neurons (Markam and Bourguignon et al, to be submitted). Together, these evidence led to the investigation of the role of these neurons in infradian sleep:wake rhythm generation. While much of the previous work on the DO involved the clock-deficient Bmal1KO mouse model, we shifted our focus on its function in the presence of the circadian clock as it better represented the dynamic interaction between the two oscillators along with the sleep homeostat in driving the sleep-wake rhythms in animals. Our findings that the NAc projecting VTA DA neurons are critical for 2ndC

emergence and infradian rhythm generation solidifies this DA population as the neural locus of the oscillator. Notably, upon 2ndC induction, we frequently observed that daily running wheel activity was under predominant control of the DO. This was very evident under 48hr cycling conditions, where some animals showed little to none running wheel activity on alternating days suggesting that the circadian clock's ability to drive daily arousal was greatly impaired, at least on intervening days with overall low activity. On the other hand, the alternating days featured extended running wheel activity reaching well into the time of day where the circadian clock typically dictates rest. Based on these observations, it can be concluded that the DO is an arousal oscillator that is able to fully override circadian sleep:wake drive. Our other unpublished work further suggest that the DO is also able to override the sleep homeostat which dictates sleep need, as we found that when the DO adopts a 48hr period the total sleep based on passive infrared motion analysis is reduced by ~30% per 48hrs (Markam and Bourguignon et al, to be submitted). Thus, the DO seems to represent an arousal oscillator that is able to greatly diminish or even suspend sleep:wake control by the sleep homeostat as well as the circadian clock. The proposed location of this arousal/wake driving oscillator in the NAc projecting VTA neurons is consistent with the finding that optogenetic activation of the DA neurons of the VTA as well as their NAc afferents was found to led to increase in wakefulness and suppression of NREM even at times of high sleep pressure (Eban-Rothschild et al., 2016a). The suggestion that the DO overrides the sleep homeostat as well as the circadian clock is perhaps also supported by the evidence that DA chiefly mediate rhythmic oscillator output (this work and (Blum et al., 2014)) given that psychostimulants acting on the DAT are known to override sleep need at least temporally and given that

GWAS studies have found significant associations between polymorphisms in the DAT and DRD2 gene loci and sleep length (Rhodes et al., 2019).

In our present study, we provide strong support for the view that NAc-projecting DA neuron of the VTA are necessary for 2ndC induction/infradian rhythm generation but what is the evidence that they are essential components of the DO or even harbor it? Elimination of DO rhythm generation can theoretically also be accomplished by disruption of the DO oscillator output pathway towards sleep:wake regulating centers, i.e., the NAc-projecting DA neurons could serve as mere mediators of this output of a DO reside elsewhere in the brain unless these DA neurons would be part of the output pathway, then manipulating them should not affect the oscillator period. That is unless they feedback onto the oscillator which would make them part of the oscillator much like the cryptochrome and period genes which are under control of the core clock transcription factors Bmal1/Clock. The proteins these genes encode feedback onto Bmal1/Clock and their manipulation affects e.g., circadian clock speed, which is not the case of other Bmal1/Clock driven genes that are not known to feedback onto Bmal1/Clock. That's why PERs and CRYs are considered clock components and other Bmal1/Clock targets such as arginine vasopressin (Mieda et al., 2015) not. Now with regard to VTA DA neurons that project to the NAc, our chemogenetic activation data indeed suggests that their manipulation can lead to changes in locomotor period arguing that they indeed either harbor the DO or represent one of its components.

Could the NAc-projecting DA neurons be part of the input pathway(s) to the actual DO? It seems conceivable that these DA neurons simply mediate the 2ndC inducing Meth signal onto the DO, which resides downstream. The neuron's ablation would then

eliminate Meth ‘signalling’ to enable 2ndC emergence. Two findings argue against such scenario, first, we have previously demonstrated that extracellular DA levels in the striatal/NAc region fluctuate in synchrony with locomotor rhythms (Blum et al., 2014) and more recently (Markam, Bourguignon et al., Submitted) also found intracellular calcium in DA neuronal projections in the NAc to fluctuate in synchrony with locomotor activity rhythms in the absence of Meth. An input pathway should not produce rhythms in the absence of the input cue (Meth) unless it is part of the output or the oscillator itself. As being part of the output is unlikely as discussed above, the collective evidence to date point to the NAc-projecting VTA neurons or a subset of them as sites or components of the DO.

This thesis has focused on identifying the neuronal substrate for the DO as it drives infradian rhythms. While molecular signatures have been provided for rhythm generation in the absence of Meth in the form of fluctuating extracellular DA (Blum et al., 2014) and intracellular calcium in the NAc (Markam, Bourguignon, et al., Submitted), we have yet to demonstrate such rhythmic molecular signatures when the animals are exhibiting infradian rhythms. Additionally, the rhythmic changes in intracellular calcium and DA levels in the DA terminals of the NAc was reported in the ultradian Bmal1KO animal in the absence of Meth. We do not know if these rhythmic changes will indeed follow the locomotor period lengthening under Meth, upon DAT disruption or chemogenetic activation. We also do not know if there will be a loss of calcium rhythmicity in mice deficient of producing DA in the VTA. Moreover, we have also not investigated whether ultradian locomotor rhythms persist when VTA DA neurons are ablated, which would require their ablation in clock-deficient mice and their behavioral monitoring in constant

darkness (Blum et al., 2014). Notably, the common vole which serves as model for robust ultradian rhythm generation in rest and activity (Gerkema & van der Leest, 1991) was shown to lose ultradian rhythmicity upon lesioning of the hypothalamic, arcuate nucleus/retrochiasmatic area (Gerkema & Daan, 1990). The arcuate nucleus is known to harbor dopaminergic neurons that project to the median eminence/pituitary gland and which have been shown to be involved in the regulation of energy homeostasis in mice (Zhang & Van Den Pol, 2016). Given that these arcuate nucleus DA neurons likely harbor the very same DA production and release machinery as the VTA DA neurons (Björklund & Dunnett, 2007), it seems plausible that they might as well act as rhythm generators, possibly contributing to ultradian locomotor rhythms generation. Thus, further investigations into the role of DA populations other than the VTA DA neurons is warranted to obtain a comprehensive picture on the role of the dopaminergic system in sleep-wake/arousal rhythm generation.

7.2.2 Mechanistic basis of the DO

The current knowledge suggests that DA has a core role in DO rhythm generation not only as an output but possibly also as an oscillator component, given that NAc/striatal extracellular concentrations of DA correlate with ultradian locomotor period (Blum et al., 2014). However, the mechanism of DO rhythm generation remains obscure. We demonstrate that tyrosine hydroxylase (TH), the rate limiting enzyme in DA synthesis is necessary for DO to function. Intriguingly the enzymatic activity of TH is depended on its phosphorylation state and feedback inhibition by dopamine (Dunkley et al., 2004). DA has a high affinity for TH and it can irreversibly bind to TH resulting in enzymatic deactivation while phosphorylation of TH can lower DA affinity through conformational change

induction (Dunkley et al., 2004). It is thus possible that the DO rhythms generation mechanism involves differential regulation of TH.

If DA inhibition of TH is indeed critical for DO function, it would further argue that changes in intracellular DA and its feedback on TH are of prime importance for rhythm generation. This can be tested by treating VTA TH KO animals with L-dopa and then with Meth. As their VTA DA neurons will be able to produce DA from L-dopa, Meth should be able to enable release of this DA upon DAT transport reversal. However, if TH is indeed a critical component of the DO, these mice should not produce a 2ndC in response to Meth.

7.3 Implications in psychiatric disorders

The sleep homeostat and the circadian clock together are thought to shape the daily cycle of sleep and wake (Borbély et al., 2016). Both processes ensure that the same amount of sleep occurs at the same time each day resulting in a rather invariable sleep-wake pattern from day to day in the absence of environmental pressures such as in case of humans, social constrains (Deboer, 2018). However, there are rhythmic sleep aberrations reported in humans that are difficult to attribute to circadian clock dysfunction. For example, patients with a bipolar disorder were reported to exhibit 48hr cycling in sleep and wake rhythms along site 48hr cycling in mood, i.e., these patients were transiting from mania to depression from one day to the next with hyperactivity associated with mania and hypoactivity with depression (Wehr et al., 1982; Welsh et al., 1986). These patterns are similar to the 48hr rhythm in rest and activity in mice exposed to high drinking water concentrations of Meth as reported here. Notably, changes in mood related behaviors during 48hr cycling have not been reported yet in mice. Intriguingly, such infradian sleep wake aberrations can be corrected to 24hr periodicities in both mice and

humans by antipsychotics suggesting that the activity patterns are driven by the same mechanisms (Fig. 1.2) (Blum et al., 2014; Tashiro, 2017). Given that our work indicates a critical role of VTA-NAc DA circuitry in inducing 2ndCs and given the observation of rhythmic changes in intracellular calcium in DA processes in the NAc (Markam and Bourguignon et al, to be submitted) as well as rhythmic changes in extracellular DA in the NAc/striatal region (Blum et al., 2014), it seems conceivable that the DO specifically resides in the NAc terminals of VTA DA neurons. If the DO is also at play in cycling bipolar patients, then the expectation would be that the NAc should show activity changes in synchrony with the patient's mood cycles. Indeed, a case study on a rapid-cycling bipolar patient who cycled in and out of a state of mania in synchrony with her menstrual cycle and at great regularity met this expectation. This patient showed increased fractional anisotropy, a measure of microstructural changes, in the nucleus accumbens among a few other adjacent structures during the manic state, when compared to MRI scans conducted in the week prior or post the 2 week period of mania (Matsuoka et al., 2014). A very recent PET study employing a radio-ligand for DAT further supported not only a central role of the NAc region in the disorder but specifically extracellular DA. Patients with current mania exhibited reduced DAT availability in the putamen and NAc compared to patients with remitted mania or healthy controls (Yatham et al., 2022). The observed lower DAT availability can be interpreted as reflection of increased extracellular DA levels in the NAc in the manic state. These studies provide support for a role of a DO in bipolar cycling that resides in DA terminals of the NAc and more generally a role of the NAc as a central mediator of the diseased state.

7.4 Future directions

While our work provides a neural basis for the DO and sheds new light on the mechanistic basis of the oscillator, there are several unanswered questions that emerged from this study.

To further demonstrate sufficiency of NAc projecting DA neurons in driving a 2ndC, one could selectively disrupt the DAT gene in the VTA or NAc-projecting DA neurons or deliver a retrograde traveling Cre-expressing virus into the NAc in combination with injection of an AAV encoding a Cre-activatable chemogenetic activator in the VTA. Both approaches chemogenetic activation and selective DAT disruption should lead to period lengthening if indeed the NAc-projecting DA neurons are sufficient for 2ndC induction. Furthermore, VTA DA neuron or NAc-projecting DA neuron ablation should be conducted in Bmal1KO mice to test if these neurons have a role in spontaneous ultradian rhythm generation.

To further our understanding of the oscillatory mechanisms of the DO, future studies may focus on the role of TH phosphorylation in 2ndC production. Furthermore, intracellular calcium recording in the DA terminals of the NAc in TH disrupted mice would establish a causal relationship between calcium fluctuations and DO action and could be used similarly to PER:Luc for furthering our understanding of the molecular dynamics of the oscillator.

Finally, inspecting sleep and mood related parameters associated with BD patients in different phases of Meth mediated 48hr rhythmicity in mice would be critical in establishing Meth-treated cycling mice as model for BD cyclicity. Ultimately, a full understanding of DO biology and its interplay with the circadian clock and the

environmental light:dark cycle may result in novel chronotherapeutic approaches for the treatment of BD and/or the reduction of BD risk.

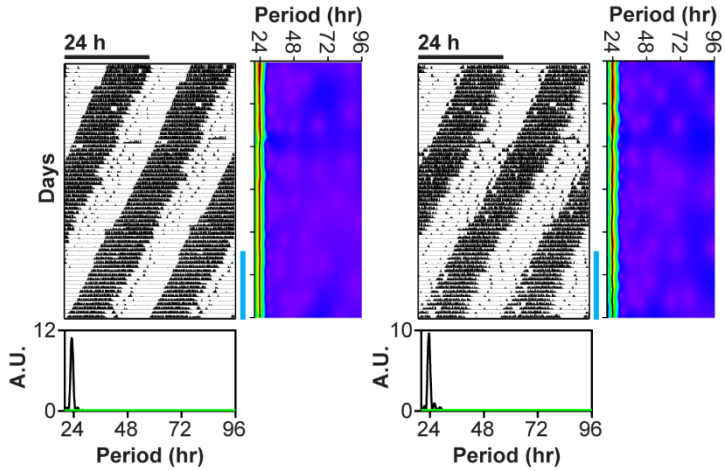
Chapter 8: Concluding remarks

Here we aimed at identifying the molecular and neural basis of infradian sleep-wake/arousal rhythms and to further our understanding of the link between the underlying oscillator process and aberrant sleep-wake rhythms associated with bipolar disorders.

To that end, we demonstrated that the recently discovered dopaminergic oscillator has an extremely wide tuning range with period reaching far into the infradian range. We also found that infradian rhythm generation relies on dopamine neurons of the ventral tegmental area that project to the nucleus accumbens. Moreover, we showed that the ability of dopamine production by these neurons is critical for infradian rhythm generation further underscoring that the underlying oscillator is dopaminergic in nature. However, we also found further evidence in support of the view that while dopamine release is of critical importance for oscillator function the mode by which dopamine exits the VTA DA neurons is not.

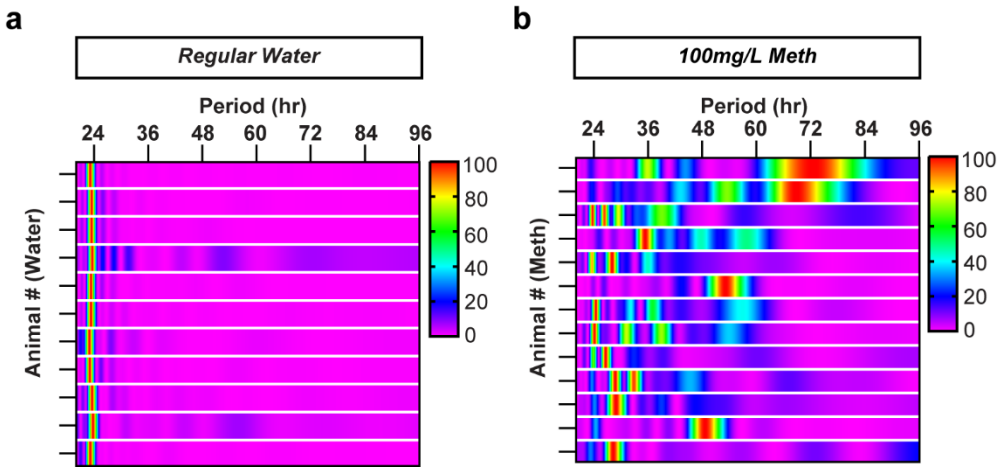
We expect that the advance this work provides on the biology of this oscillator process, which together with the circadian clock can shape the daily sleep-wake pattern, will critically further our understanding of infradian rhythms biology but also human sleep pathologies and the etiology of psychiatric conditions with cyclical characteristics such as bipolar disorder.

Appendix I Supplementary figures



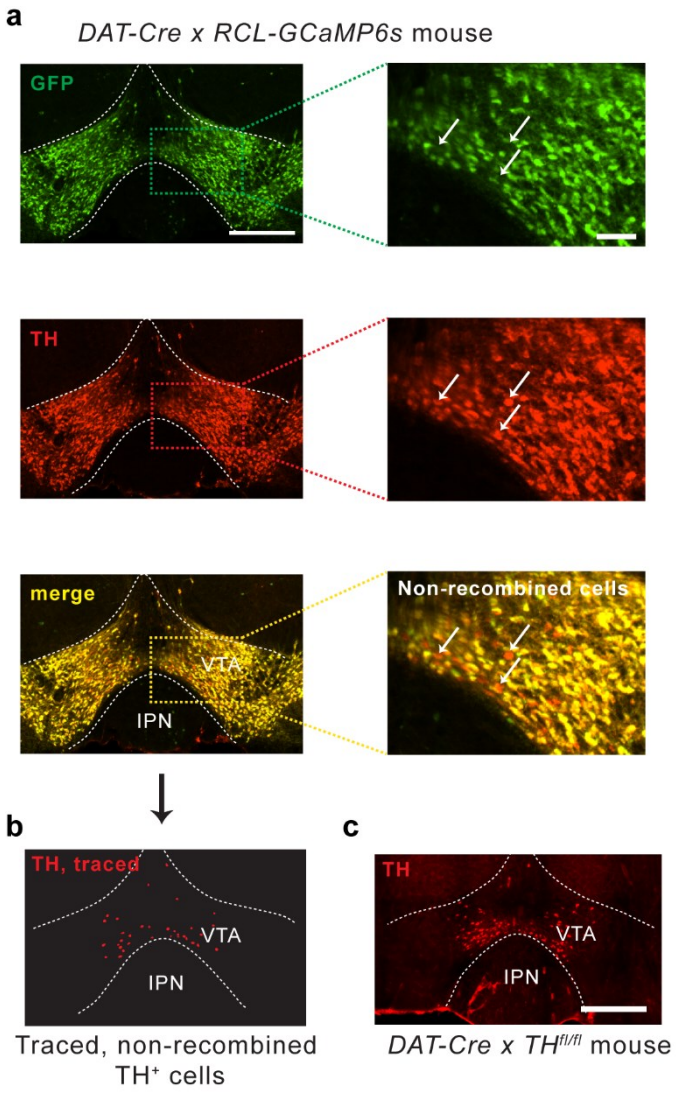
Supplementary figure 3.1: Activity rhythms under water.

Representative actograms displaying running wheel activity patterns of two single housed animals. Besides each actogram is the continuous wavelet transforms heatmap showing amplitudes of oscillation and the black trace indicates the ridge of the local amplitude maxima. Bottom: Lomb-Scargle periodogram corresponding to the14 day time span indicated by blue bar



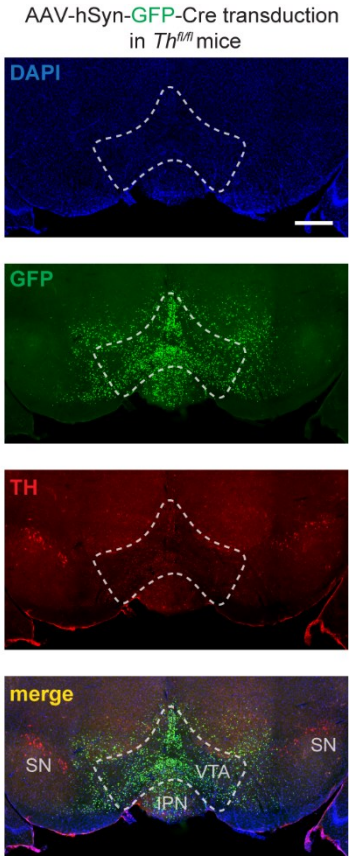
Supplementary figure 3.2 : LS periodogram heatmaps, Water vs Meth

Individual periodograms normalized and plotted as a heatmap from records of animals supplemented with **a)** Water, **b)** 100mg/L Meth



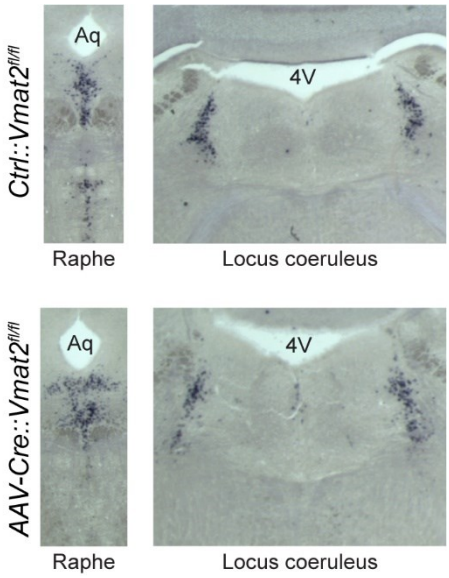
Supplementary figure 4.1 : Cre activity in the VTA of the DAT-Cre mouse line.

a) Some of the TH+ cells that are preferentially located in the ventral VTA apical to the IPN were found to lack GCaMP6s expression in DAT-Cre x RCL-GCaMP6s mice. Shown are immunofluorescence images of the VTA stained with antibodies against TH and GFP, respectively. Images on the right show enlargements of the boxed areas of the images on the left, **b)** Selective display of non-recombined (GFP-negative) cells that were manually identified in **a**. **c)** TH immunostaining in the VTA of a DAT-Cre x $TH^{fl/fl}$ mouse. IPN, interpeduncular nucleus. Scale bar, Scale bars, 500 µm (A, left) and 100 µm (A, right).



Supplementary figure 4.2 : GFP tagged cre expression in the VTA

Midbrain GFP/TH immunofluorescence after bilateral injection of AAV-GFP-Cre into the VTA of a $TH^{fl/fl}$ mouse. GFP positive nuclei indicate that virus spread was largely limited to VTA sparing the SN



Supplementary figure 6.1 : Spared Vmat2 in the LC and Raphe

In situ hybridization showing preservation of Vmat2 signal in raphe nucleus and locus coeruleus in VTA Vmat2KO mice (lower) versus controls (upper).

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