Effects of Methamphetamine on Circadian Rhythms of Mice

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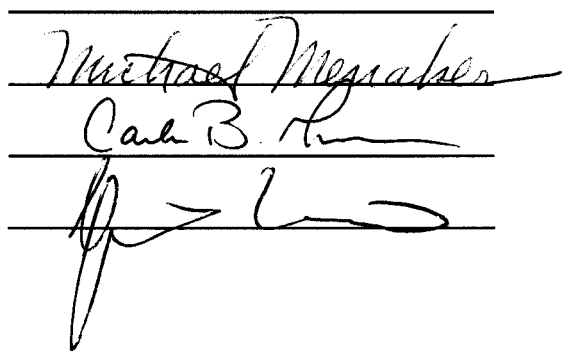

Abstract

The circadian system allows an organism to anticipate rhythmic changes in the external conditions and thereby increases its fitness. In rodents, the circadian clock resides in a pair of nuclei called the suprachiasmatic nuclei (SCN) which regulates rhythms of locomotor behavior, body temperature and some other physiological variables. However, this hierarchical organization of the circadian system is challenged by the discovery of two SCN-independent major oscillators; a feeding-entrainable oscillator (FEO) and a methamphetamine-sensitive circadian oscillator (MASCO). The presence of SCN, FEO and MASCO suggests a central multi-oscillatory organization of the circadian system in rodents, similar to the organization found in non-mammal vertebrates. At present, we know very little about how this complex multi-oscillator system is integrated.

Although there is substantial information available about the SCN, the molecular components, localization and natural role of MASCO in the body are unknown. In fact, it is still debated whether MASCO is a *bona fide* circadian oscillator rather than a simple hourglass mechanism. This is largely due to the lack of a robust circadian model to study the effects of methamphetamine (MAP) on circadian rhythms. This thesis aims to fill some of these gaps in our understanding of this fascinating major oscillator

Using mice, we successfully replicated the published effects of chronic MAP on the circadian rhythms of rats. In fact, the differences such as more robust and clear activity rhythms, more pronounced MAP-induced second components of the rhythm and obvious sex and strain differences make the mice a better model. The results also indicated that MASCO interacts with the SCN in a manner that is strain, sex and dose dependent. Our experiments testing the hourglass explanation showed that the effects of MAP can not be explained by such a mechanism. This suggests that MASCO has circadian properties and is indeed a *bona fide* circadian clock. We also tried to elucidate the similarities between the molecular components of SCN and MASCO using several circadian mutant mice that were available to us. Although, the results of these experiments are not adequate to propose a list of known circadian genes that are involved in MASCO function, they provide the background for future studies that will determine the differences between the molecular components of SCN and MASCO.

Utilization of the mouse strains presented in this thesis will provide an easy to obtain standard model where MASCO can be studied using latest developments in genetics and molecular biology. Identification of the natural role of MASCO in the body and its interactions with other oscillators in the body is of both scientific and clinical significance and will greatly improve our understanding of the circadian system in mammals.

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I would like to thank Dr.Menaker for his continuous support and outstanding mentoring. I owe whatever I accomplished during my time at UVA to him and could never repay my debt. I am very lucky for having the chance to witness his wisdom in science and life. I am honored to be his student and I will do my very best to become a member of the great scientists who were once his student. I will greatly miss our inspiring “10 minute” discussions and his post-it notes on my desk. I believe our paths will cross in the future and I will again greatly enjoy science with him.

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Chapter 1: Introduction

Partially published in (Davidson, Tataroglu et al. 2005)

(I contributed the literature review on the effects of MAP and its discussion to this review paper)

Circadian system in mammals:

The circadian system allows an organism to anticipate rhythmic changes in the external conditions and thereby increases its fitness. Accurate anticipation of dawn and dusk, for example, helps a nocturnal animal to avoid its diurnal predators. It also provides a safe window of opportunity for activities such as foraging, hunting or breeding. In plants, the circadian system provides cues that synchronize the opening and closing of leaves. Although the day-night cycle is the dominant environmental synchronizer for most organisms, many other aspects of the environment such as temperature, humidity and activity of the predators/preys are also rhythmic.

In rodents, the circadian clock resides in a pair of nuclei called the suprachiasmatic nuclei (SCN) located dorsally to the optic chiasm in the brain. SCN regulate rhythms of locomotor behavior, body temperature and some other physiological variables. The light information that is received by specialized retinal photoreceptors is conveyed to the SCN via the retino-hypothalamic tract (RHT) where it resets an oscillating molecular feedback loop that underlies the rhythmic output of this clock. According to the current model of this feedback mechanism: *Bmal1* and *Clock* genes encode proteins that heterodimerize in the cytoplasm and translocate into the nucleus where they activate the transcription of PERIOD1 (PER1), PERIOD2 (PER2), PERIOD3 (PER3), CRYPTOCHROME1 (CRY1) and CRYPTOCHROME2 (CRY2) proteins. PER and CRY proteins form

a complex in the cytoplasm and translocate into the nucleus where they inhibit the activity of the BMAL1/CLOCK heterodimer; hence inhibiting their own transcription. BMAL1/CLOCK heterodimer also enhances the transcription of REVERB-alpha which feeds back onto *Bmal1* and reduces its transcription. Another circadian protein found in the forebrain, NPAS2, can functionally substitute for CLOCK and form BMAL1/NPAS2 heterodimer which can also activate transcription of *Per* and *Cry* genes. CASEIN KINASE I-epsilon (CKIe) binds and targets PER protein for degradation through the ubiquitination-mediated degradation pathway (for a full review see (Lowrey and Takahashi 2004).

Similar molecular feedback loops are also found in the peripheral tissues such as liver, heart, kidney, ovary and salivary glands. It is believed that these “peripheral clocks” are continuously synchronized (entrained) via unknown neuronal and hormonal signals coming from the SCN (Davidson, Yamazaki et al. 2003).

This hierarchical organization of the circadian system, however, is challenged by the discovery of two SCN-independent major oscillators; a feeding-entrainable oscillator (FEO) and a methamphetamine-sensitive circadian oscillator (MASCO)(Honma, Honma et al. 1987; Honma, Honma et al. 1989; Davidson, Tataroglu et al. 2005; Tataroglu, Davidson et al. 2006). The presence of SCN, FEO and MASCO suggests a central multi-oscillatory organization of the circadian system in rodents, similar to the organization found in non-mammal

vertebrates (Davidson, Tataroglu et al. 2005). Furthermore, there are many other circadian oscillators in peripheral organs, tissues and cells. At present we know very little about how this complex multi-oscillator system is integrated: Which oscillators influence which others? Are they coupled through neural, humoral or behavioral links? What is the adaptive significance of their phase relationships, and what are the consequences of the loss of internal synchrony? The interactions between these oscillators in the body are important for understanding how the mammalian circadian system is organized.

Although there is substantial information available about the SCN, the molecular components, localization and natural role of MASCO in the body are unknown. In fact, it is still debated whether MASCO is a *bona fide* circadian oscillator rather than a simple hourglass mechanism(Ruis, Buys et al. 1990). The experiments presented in this thesis aim to fill some of these gaps in our understanding of this fascinating major oscillator.

Methamphetamine (MAP):

Methamphetamine (MAP) is a drug of abuse chemically similar to amphetamine and adrenaline. However, MAP has fewer peripheral actions than amphetamine and enters the brain more rapidly due to its chemical structure. After oral consumption, MAP is quickly absorbed from the gastrointestinal tract. It is metabolized to amphetamine and several other metabolites in the liver via

aromatic hydroxylation, N-dealkylation and deamination and excreted in the urine. Elimination half-life of MAP after repeated injections is around 70 minutes in rats and 12 hours in humans(Cho, Melega et al. 2001). Short term effects include increased release of dopamine (and noradrenaline) from the nerve terminals due to inhibition of re-uptake and monoamine oxidase (MAO). Long term administration of MAP results in toxicity to dopaminergic, and to lesser extent serotonergic and adrenergic neurons. The exact mechanism of this toxicity is still unknown, but increased local temperature and increased production of free radicals is thought to be involved(Itzhak and Achat-Mendes 2004).

Effects of MAP on circadian behavior (and other) rhythms:

Chronic application of MAP reversibly increases the total daily activity, length of "alpha" (the duration between the onset and offset of daily activity) and the period length of locomotor activity rhythms of rats (Honma, Honma et al. 1986). Three-fourths of rats held in a 12/12 light/dark (LD) cycle and given chronic MAP show a second rhythmic component of locomotor behavior, the period length of which is longer than 24 h and which exhibits relative coordination with the main component which is entrained to the LD cycle (Honma, Honma et al. 1986). In addition, MAP induces robust activity rhythms in SCN-lesioned (SCNX) arrhythmic rats that occasionally persists for several cycles after withdrawal of MAP(Honma, Honma et al. 1987; Honma, Honma et al. 1988; Honma,

Kanematsu et al. 1992). Similar rhythms can also be induced in SCNX rats when MAP is administered via osmotic pumps instead of drinking water(Honma, Honma et al. 1987). In SCNX rats, chronic MAP also induces circadian rhythms of body temperature, drinking, feeding, total activity and corticosterone (Morimasa, Wirz-Justice et al. 1987; Rietveld, Korving et al. 1987; Honma, Honma et al. 1988; Ruis, Buys et al. 1990; Hiroshige, Honma et al. 1991). These rhythms seem to preserve their normal phase relationships with the locomotor activity rhythm during the MAP treatment.

These data suggest that MASCO is anatomically distinct from the SCN and manifests itself in routinely measured circadian markers. Alternatively, it is possible to explain these results (with the important exception of rhythmicity produced in SCNX animals treated with MAP in osmotic pumps) by an hourglass mechanism that is driven by rhythmic drinking(Ruis, Buys et al. 1990). Because MAP is known to cause hyperactivity and increase the duration of wakefulness (alpha), this spontaneous increase in alpha, followed by increased rebound sleep may seem like a lengthened rhythm. However, such an hourglass mechanism requires rhythmic presence of the stimuli. Therefore, the persistence of rhythms after withdrawal of MAP in SCNX rats and the induction of rhythms by MAP given via osmotic pumps suggest that the hourglass explanation is unlikely. However, there are several problems with these previously published experiments that should be resolved before concluding that the MAP phenomenon is not the result of an hourglass mechanism but indeed of a circadian process. First, not all

investigators have been able to repeat these results (unpublished data, personal communications). Second, the osmotic pump experiment has never been successfully replicated and the published experiment used just three rats. Thus, the possible explanation of the effects of MAP by an hourglass mechanism driven by an underlying drinking rhythm has never been fully tested.

Localization of MASCO:

The location of MASCO is unknown. However, there are several studies that show possible involvement of certain brain areas and neurotransmitters in MASCO function. Although, MASCO is probably localized in the brain, it is almost certainly not in the retina, since enucleation does not abolish the rhythmicity of SCN-lesioned, methamphetamine-treated rats (Rietveld, *et al.*, 1987).

Both dopamine and serotonin systems seem to be involved in the circadian effects of MAP(Honma and Honma 1995). Injections of Haloperidol, which is a dopamine antagonist with some sedative effects, cause phase-shifts of the MAP-induced circadian activity rhythms in SCN-lesioned arrhythmic female rats. In contrast, Pentobarbital or ether, which also has similar sedative effects, do not cause phase-shifts(Honma and Honma 1995). This suggests that the dopaminergic system is either involved in the entraining pathway of MASCO or in MASCO itself. However, since Haloperidol is also known to affect 5-HT and

noradrenergic systems, the possible involvement of these neurotransmitters can not be excluded.

A single injection of MAP was shown to induce *Period1* gene expression in the Piriform cortex (PC) and Caudate-Putamen (CPU) in the mouse brain, but not the SCN (Nikaido, Akiyama et al. 2001). In addition, chronic MAP shifts the phase of circadian expression of *Period1, Period2* and *Bmal1* mRNA in CPU and Parietal cortex (PtA) and induces *Clock* mRNA expression in CPU of rats (Masubuchi, Honma et al. 2000). Although authors of both studies suggested that these brain areas (especially CPU) may be the site of MASCO, it is also possible that they may simply be on the output pathway of MASCO and are affected by its activity. Furthermore, it is still unknown whether the measured circadian genes (such as *Period* or *Clock*) are even involved in MASCO. This requires administration of MAP to mice that carry mutations in these circadian genes.

Another approach to localization would be lesion studies. Unfortunately, lesions cannot be performed on all these areas since some of them are quite large (such as CPU) and are vital for normal functioning of the brain. However, some of them (such as the PtA) can be lesioned and the others can be studied in slice culture. In addition, transgenic animals can be made using region specific promoters (for example a CPU-specific promoter in combination with a doxycycline system) that are combined with conditional toxicity inducing agents. A temporary shut down of

these areas can be achieved this way and their involvement in MASCO function can be studied.

MAP-induced rhythms can be entrained by restricted-feeding regimes which suggests that MASCO and FEO may share components or even be the same clock (Hiroshige, Honma et al. 1991; Honma, Kanematsu et al. 1992). FEO was suggested to be localized in the nervous system and if this is true then MASCO (or at least its entraining pathways) might also be in the brain.

Molecular components of MASCO:

Like many of its features, the molecular components of MASCO are also unknown. In the only published experiment addressing this question, authors applied MAP to arrhythmic $Clock^{m1Jt}$ mutant mice and showed that chronic MAP administration induces robust wheel running rhythms that persist even after withdrawal(Masubuchi, Honma et al. 2001). Although the authors suggest that CLOCK is not required for MASCO function, this experiment can not rule out involvement of CLOCK in MASCO. NPAS2 is known to be a functional substitute for CLOCK (Reick, Garcia et al. 2001) and MASCO might be utilizing this protein in the absence of CLOCK while CLOCK may still be involved when it's present. In addition, $Clock^{m1Jt}$ mutant mice are known to have a weak phenotype. A simple cage change has been shown to induce stable rhythmicity in arrhythmic $Clock^{m1Jt}$ mutant mice. Furthermore, these mice still possess a CLOCK protein

(although mutated) and there is no reason to assume that the effects of MAP have to depend on the mutated domain of the protein. It should also be noted that the authors have not observed multiple components of the rhythm in these intact Clock^{m1Jt} mutant mice. If we assume that the longer period length component of the rhythm represents MASCO output, this suggests that the mutation altered MASCO function so that the secondary components of the rhythm are no longer observed; hence CLOCK protein may be required for MASCO or its output pathways.

Npas2^{tm1Slm}/ Clock^{m1Jt} double-mutant mice or *Clock* null mice have to be tested for the effects of MAP to address the involvement of these proteins. Furthermore, it has been recently suggested that a *Clock* null mutation has a mild circadian phenotype; hence its specific role in the circadian clockwork is not completely clear(Debruyne, Noton et al. 2006).

Elucidating the molecular components of MASCO is important for understanding the differences and similarities between MASCO and SCN. In addition, such studies may provide new molecular markers for localizing MASCO. For example, if NPAS2 is found to be involved in MASCO, but CLOCK is not, it is reasonable to search for MASCO in the forebrain where NPAS2 is expressed. Furthermore, if MASCO and SCN share only a portion of the known circadian genes, it would suggest that there are unknown proteins that interact with the known circadian genes.

Chapter 2: The Methamphetamine-Sensitive Circadian Oscillator (MASCO) in Mice

Published in (Tataroglu, Davidson et al. 2006)

(I performed all the experiments and wrote this paper with the help of Prof. Michael Menaker and Dr. Alec Davidson)

Abstract:

The suprachiasmatic nucleus (SCN) orchestrates synchrony among many peripheral oscillators and is required for circadian rhythms of locomotor activity and many physiological processes. However, the unique effects of methamphetamine (MAP) on circadian behavior suggest the presence of an SCN independent, methamphetamine-sensitive circadian oscillator (MASCO). Substantial data collected using rat models show that chronic methamphetamine dramatically lengthens circadian period of locomotor activity rhythms, and induces rhythms in animals lacking an SCN. However the anatomical substrate and the molecular components of the MASCO are unknown. The response to MAP is less well studied in mice, a model that would provide the genetic tools to probe the molecular components of this extra-SCN oscillator. We tested the effects of chronic MAP on 2 strains of intact and SCN-lesioned mice in constant dark and constant light. Furthermore we applied various MAP availability schedules to SCN-lesioned mice in order to confirm the circadian nature of the underlying oscillator. Our results indicate that this oscillator has circadian properties. In intact mice the MASCO interacts with the SCN in a manner that is strain, sex and dose dependent. In SCN-lesioned mice it induces robust free-running locomotor rhythmicity which persists for up to 14 cycles after methamphetamine is withdrawn. In the future, localization of the MASCO and characterization of its underlying molecular mechanism as well as its interactions

with other oscillators in the body will be essential to a complete understanding of the organization of the mammalian circadian system.

Introduction:

The circadian time-keeping system increases the fitness of an organism by enabling prediction of regularities in the environment. Rhythmic stimuli such as the light/dark (LD) cycle entrain the circadian system to the external environment. In rodents, light information received by the retina is conveyed to the suprachiasmatic nucleus (SCN) in the brain through the retino-hypothalamic tract (RHT) where it resets the molecular feedback loop that underlies the rhythmic output of this pacemaker (for review see (Mistlberger 2005). It is widely believed that the SCN is the main pacemaker that orchestrates synchrony among the circadian oscillators which are present in almost all tissues, and is chiefly responsible for the generation of rhythms in locomotor activity. However, the completeness of this model of mammalian circadian organization is challenged by the fact that under some circumstances animals with SCN lesions display robust circadian rhythms of behavior. One such circumstance is chronic administration of methamphetamine (MAP). The results of experiments in which MAP is chronically administered in the drinking water have led to the hypothesis that the brain contains an SCN-independent, methamphetamine-sensitive circadian oscillator (MASCO) (Honma, Honma et al. 1987; Hiroshige, Honma et

al. 1991; Davidson, Tataroglu et al. 2005). The exact location and the molecular mechanism of the MASCO are unknown.

The effects of MAP on circadian behavior were first described in rats in 1986 by Honma and colleagues (Honma, Honma et al. 1986). They showed that chronic application of MAP in the drinking water reversibly increases the amount of total daily activity, the length of alpha (the duration between the onset and offset of daily activity) and the period length of the activity rhythm (Honma, Honma et al. 1986). These effects persist in constant darkness and disappear after withdrawal of MAP(Honma, Honma et al. 1986). Three-fourths of rats held in a 12/12 light/dark (LD) cycle and given chronic MAP show a second rhythmic component of locomotor behavior, the period length of which is longer than 24 h and which exhibits relative coordination with the main component which is entrained to the LD cycle (Honma, Honma et al. 1986). MAP also induces robust activity rhythms in SCN-lesioned (SCNX) rats that occasionally persist for several cycles after withdrawal of the drug (Honma, Honma et al. 1987). Chronic MAP produces rhythmicity in arrhythmic $Clock^{m1Jt}$ mutant mice in constant darkness (DD) (Masubuchi, Honma et al. 2001). Chronic MAP also induces circadian rhythms of body temperature, drinking, feeding, total activity and corticosterone (Morimasa, Wirz-Justice et al. 1987; Rietveld, Korving et al. 1987; Honma, Honma et al. 1988; Ruis, Buys et al. 1990; Hiroshige, Honma et al. 1991). These data demonstrate that the MASCO is anatomically distinct from the SCN.

It has been proposed that an hourglass mechanism rather than a circadian oscillator may explain MAP-induced rhythmic activity (Ruis, Buys et al. 1990). According to this model, spontaneous drinking of MAP can result in a lengthened activity bout, followed by sleep. After awakening, the animal drinks again, thereby restarting and reinforcing the cycle. This model predicts that if the animals were prevented from rhythmically ingesting the drug, the rhythms would not be evident. To address this possibility, Honma et al. implanted arrhythmic SCN-lesioned rats with osmotic pumps filled with MAP and observed circadian rhythms in locomotor activity (Honma, Honma et al. 1987). However, there are limitations to what can be concluded from this experiment. Data from two rats were shown and both exhibited masking by the LD cycle in which they were housed, suggesting that their SCN may not have been completely lesioned. To date there is no other evidence for the generation of circadian rhythms by MAP in previously arrhythmic animals that does not depend on the rhythmic consumption of MAP. Therefore the hourglass hypothesis still warrants investigation.

While Masubuchi et al. showed MAP-induced rhythms in $Clock^{m1Jt}$ mutant mice (Masubuchi, Honma et al. 2001), the potential use of mice in the study of circadian effects of MAP has not been systematically explored. To pursue this potentially very useful model, we evaluated the effects of MAP on rhythmicity of C57BL/6 and C3H mice. Our results show that C57BL/6 mice exhibit all the behavioral responses to MAP that were previously observed in rats, in some cases much more clearly; C3H mice also respond to MAP but less robustly. We

demonstrate that in mice there is indeed a *bona fide* MAP-sensitive circadian oscillator –not an hourglass- that underlies the effects of MAP on circadian behavior. The MASCO interacts with the SCN when it is present, and the strength of this interaction is strain, sex and dose dependent.

Methods:

Animals and housing:

8-20 week old male and female mice were individually housed in a temperature, humidity and light intensity controlled environment; wheel-running behavior was recorded using the Clock lab computerized data collection system (Actimetrics, Evanston, IL). Mice were either kept in 12/12 light/dark, constant light (112-1976 lux) or constant darkness. C57BL/6 mice were purchased from Harlan (Indianapolis, IN), Taconic (Hudson, NY) or Jackson laboratories (Bar Harbor, ME). Transgenic *Period1-luciferase* (*Per1-luc*) (Lundkvist, Kwak et al. 2005) mice on congenic C57BL/6 background and C3H mice (wild type at the *retinal degeneration* (*rd)* locus) were raised in animal facilities at the University of Virginia under conditions approved by the Animal Care and Use Committee at the University of Virginia (light, 0500-1700h; dark, 1700-0500h).

Methamphetamine and food administration:

Food, water or methamphetamine-containing water (d (+)-Methylamphetamine hydrochloride, SIGMA, #M8750) was available ad libitum unless otherwise stated. The doses of methamphetamine used were 0.0025%, 0.005%, 0.0065%, 0.0075% or 0.01% (w/v). A mouse consuming 8 ml of 0.005% MAP per day would ingest 0.4 mg/day.

SCN lesions:

Mice were kept in 12/12 LD for 10-71 days during which bilateral electrolytic lesions of the SCN were made and post-operative care was given. Lesions were made by applying 1.1 mA DC current for 20 seconds using a Teflon coated tungsten wire electrode (0.5mm exposed tip) under isoflurane anesthesia. Anterioposterior, medio-lateral and dorso-ventral coordinates relative to bregma were 0.1 mm, ±0.25 mm and -5.6 mm (respectively) for *Per1-luc mice* with 4.4-4.6 mm bregma-lambda distance; 0.3 mm, ±0.25 mm and -6.0 mm (respectively) for C57BL/6 mice with 3.7-3.9 mm bregma-lambda distance; and -0.6 mm, ±0.25 mm and -5.6 mm for C3H mice with 4.5-4.6 mm bregma-lambda distance. Animals were then released to DD for 20-53 days for confirmation of arrhythmicity using periodogram analysis. Although we did not perform histology on all the brains, pilot studies suggest that the lesions were relatively large and may have impinged on areas such as optic chiasm, para-ventricular nucleus and

ventro-medial hypothalamus. The success rate for producing behavioral arrhythmicity was 68%. Rhythmic mice were excluded from experiments.

Statistics:

Periodogram analyses were performed in the range of 19 to 34 hours using the Clock lab analysis program (Actimetrics, Evanston, IL) on at least 10 days of continuous data and summarized in Table 1. Block size was 10 minutes for all actograms. Mice that were still rhythmic after SCN-lesions or LL-treatments were excluded from analysis. Average period lengths of groups were compared using unpaired, Welch-corrected t-test and $p<0.05$ was considered significant. Phase determinations were made by fitting an estimation line to at least 6 activity onsets using the Clock lab software.

Results:

Effects of MAP on the circadian wheel running activity of intact, SCN-lesioned or LL-treated mice:

The effects of MAP on circadian wheel running activity of intact or SCN-lesioned C57BL/6 and *Per1-luc* mice on congenic C57BL/6 background were similar for all

experiments we performed. Consequently data have been combined for both strains which are referred to as C57BL/6 mice. Effects of MAP on period length are summarized in Table 1.

Effects on intact C57BL/6 mice:

Mice were kept in LD (~220 lux) for 4 days and then released to DD for 18 days. The first group of mice (n= 10 males and 9 females) received 0.005% MAP for 25 days and then 0.01% MAP for 19 days while the second group (n= 12 males and 11 females) received only 0.01% MAP for 44 days. This was followed by 15 days of withdrawal (pure water) for both groups.

0.005% or 0.01% MAP increased the amount of total daily wheel running activity, length of alpha (duration of activity) and circadian period in all intact mice within 8 days (Fig 1A). Fourteen of the 42 mice tested showed two components of the wheel running rhythm during the MAP treatment (Fig 1A) while the others showed only one (not shown). In either case, MAP reversibly increased period length of any rhythmic component ($p<0.001$). In animals showing 2 components, the period lengths of both components were significantly different from each other ($p<0.0001$). Hereafter we refer to the shorter period component as the first and the longer one as the second rhythmic component. We did not see an increase in the period length of the first component when the dose was raised from 0.005% to 0.01% ($p=0.66$). However, second components were more

frequently observed when the mice were treated directly with the higher dose of MAP (0.01%). Four of the 19 mice that received 0.005% MAP followed by 0.01% MAP showed a second component while 10 of 23 mice that were treated directly with 0.01% MAP showed this component. There was clear relative coordination between these components; the period lengths of each component changed as their phase relationships varied (Fig 1A). This interaction shifted the phase of the first component as can be seen following withdrawal in Fig 1A. All of these effects disappeared within several cycles after withdrawal. Although male mice had a slightly longer free-running period before the treatments ($p=0.024$), we observed no significant sex differences at either dose or after withdrawal.

Effects on SCN-lesioned C57BL/6 mice:

A group of SCN-lesioned C57BL/6 mice (n=15 males and 8 females) were given 0.005% MAP for 35 days in DD. MAP treatment induced robust rhythms in all SCN-lesioned C57BL/6 mice. Rhythmicity lasted as long as MAP administration continued; period length increased gradually and then became stable in all mice (Fig 1B). The average period length of the induced rhythms was 26.29±1.53 hours. Only one rhythmic component was ever observed in these mice. The average period lengths of these rhythms was similar to those of the second components observed in intact C57BL/6 mice in DD ($p=0.076$). We observed no significant sex differences in the average period length of the MAP-induced rhythm ($p=0.59$).

Effects on LL-treated C57BL/6 mice:

Intact C57BL/6 mice were kept in LL (112-1976 lux, intensity varied proportionally to the distance from the light source) for 30-37 days and, after confirmation of arrhythmicity, 0.005% MAP was administered for 21 -28 days.

Three mice (n=2 males and 1 females) became arrhythmic in LL. Robust rhythms were induced in these with an average period length of 27.44±0.20 hours (Fig 1C). Other mice (n=3 males and 5 females) remained rhythmic in LL with an average period length of 26.29±1.53 hours. MAP significantly increased the period length of the rhythm in these mice to 28.21±0.65 hours (p=0.0001, data not shown). Only one rhythmic component was observed in either group of mice.

Effects on intact C3H mice:

Intact C3H mice (n= 6 males and 5 females) were kept in DD for 18 days and then received 0.0025%, 0.005%, then 0.00625% MAP for 25, 27 and 30 days, respectively. This was followed by 20 days of pure water.

In contrast to C57BL/6, intact C3H mice showed sporadic and sex-dependent effects of MAP (Fig 2A). Three of six C3H male mice showed a longer 2nd circadian component in their behavior during the MAP treatment. However, the occurrence of this 2nd component did not correlate with the dose of MAP and did

not last for the duration of the drug treatment (Fig 2A, longest lasting example). Neither male nor female C3H mice showed any significant effects of MAP on the period length of the first component ($p=0.14$). MAP failed to induce any second components in the female C3H mice. Furthermore, we did not observe a significant increase in period length in the first component or induction of a second component that exhibited relative coordination even at higher doses (0.0075% or 0.01%, data not shown). In fact, these doses, which are tolerated without difficulty by C57BL/6 mice, proved lethal to C3H mice within 10 days.

Effects on SCN-lesioned C3H mice:

SCN-lesioned C3H (n=2 males and 2 females) mice were administered 0.0025% MAP for 14 days and then 0.005% MAP for 50 days. At the higher does, MAP induced robust activity rhythms (Fig 2B) with an average period length of 24.54±0.64 hours which sometimes changed abruptly (Fig 2B female).

Effects on LL-treated C3H mice:

Intact C3H mice (n=6) were kept in LL (112-1976 lux, intensity varied proportionally to the distance from the light source) for 65 days and after confirmation of arrhythmicity in 3 mice, 0.005% MAP was administered to all mice for 15 days. The three arrhythmic mice showed robust rhythms with an average period length of 24.63±1.27 hours during MAP administration (Fig 2C).

The other three mice stayed rhythmic in LL with an average period length of 25.22±0.82 before and 24.94±0.59 hours after MAP administration. Period length of the activity rhythms of two out of three of these mice decreased slightly during MAP administration (data not shown).

Effects of MAP presented on different schedules (tests of the "hourglass" hypothesis)

SCN-lesioned C57BL/6 mice were treated with 0.005% MAP for at least 1 month to achieve stable rhythmicity and were used in the following experiments.

Effects of a single 8 hour withdrawal of MAP:

MAP-containing water bottles of 4 SCN-lesioned C57BL/6 mice were replaced with bottles containing pure water for 8 hours at CT12, a time when mice drink a high proportion of their daily water intake. If the locomotor rhythm were being driven by an hourglass, a mechanism that would halt when its driving stimulus (MAP) is not present, then an eight hour delay in the onset of MAP availability would be expected to result in a substantial delay of the rhythm. However, this "water pulse" actually resulted in a phase-advance in all four mice tested [by 25, 165, 170 and 525 minutes (Fig 3A)]. We also observed earlier onsets for the first two cycles after the 8 hour withdrawal but this was not reflected in the phase calculations since it was transient.

Entrainment to 14/10 or 14/8 MAP/water schedules:

Six SCN-lesioned C57BL/6 mice were treated with 14 hours of MAP followed by 10 hours of water (14/10 MAP/water) for 20 days to test if the MAP-induced rhythm would entrain to this schedule immediately as expected from an hourglass. However, 3 of 5 mice showed obvious transients in their activity onsets until they entrained to the onset of the “water only” portion of the cycle (Fig 3B). The other two mice showed somewhat less organized rhythms until they entrained to this schedule within 3 days (data not shown).

Four SCN-lesioned C57BL/6 mice were placed on a 22 hour period (14/8 MAP/water) schedule for 16 days to test if this period length is outside the range of entrainment of the MAP-induced rhythm. All mice showed relative coordination to the onset of water (Fig 3C), but did not completely entrain.

Persistence of rhythms after complete withdrawal of MAP:

MAP-induced rhythms persisted after withdrawal in 3 out of 9 male and 6 out of 8 female SCN-lesioned C57BL/6 mice for up to two weeks (14 days in four mice, 4 days in two mice and 8, 9 and 12 days in others, Fig 3D). The average period length of the persisting rhythms was shorter (23.54±1.13 hours) than during MAP-treatment. All other mice became arrhythmic immediately after withdrawal (data not shown). Although, we could not perform a valid statistical comparison

(only 3 males in the sample), there were no obvious sex differences in the average period length of the persisting rhythms.

Persistence of rhythms during 24/24 MAP/water schedule:

5 SCN-lesioned C57BL/6 mice were put on a 24/24 MAP/water schedule for 32 days. Although this schedule lacked a circadian component it was able to sustain MAP-induced rhythms for longer durations than complete withdrawal (Fig 3E). Activity onsets were also observed on days of water only. The average period length of the persisting rhythm was shorter (24.30±0.74 hours) than during MAP-treatment.

Discussion:

Using mice, we have replicated and extended the pioneering work of the Honma laboratory on the effects of chronic methamphetamine on the circadian rhythms of rats (Honma, Honma et al. 1986; Honma, Honma et al. 1987). This paves the way for a genetic and molecular analysis of the methamphetamine-sensitive circadian oscillator (MASCO), the location of which is still unknown.

Our results demonstrate that C57BL/6 mice exhibit behavioral responses to chronic MAP that are similar to those of rats (Honma, Honma et al. 1987). The

similarities include MAP-induced increase in alpha and period length in intact mice, robust MAP-induced rhythms in SCN-lesioned mice, and persistence of these rhythms after withdrawal of the drug. However, there are some differences as well. In this mouse strain, we did not see significant differences between males and females, in contrast to the stronger response to MAP in female rats (Honma, Honma et al. 1986). The effects of MAP appear more quickly after administration (7-14 days in mice *vs*. 2 months in rats (Honma, Honma et al. 1986). In addition, relative coordination between the two rhythmic components observed in the intact mice is more pronounced than in rats(Honma, Honma et al. 1986). Of the two doses of MAP that we used, the higher dose induced second components in the behavior of intact mice more frequently than did the lower dose. There were no dose-dependent effects on period length.

We tested the "hour glass" model in several ways and in no case were the data consistent with it. To varying degrees, these data support the hypothesis that the mice possess an extra-SCN circadian oscillator that becomes manifest in locomotor behavior under the influence of chronic methamphetamine.

We used four different experimental paradigms to test the hour glass model. The strongest evidence against the hour glass model comes from experiments in which SCN-lesioned mice were entrained to cycles of MAP-containing water alternating with pure water (MAP/H_2O). The hour glass model predicts that under such circumstances, the mice should immediately assume the

period of the exogenous cycle and maintain a fixed phase relationship to it. They do not do so. In fact, when the period of the MAP/H_2O cycle is 24h, the mice entrain to it after many days of transients (Fig. 3B), and, even more compelling, when the period of the MAP/H_2O cycle is 22h, the mice fail to entrain, but nonetheless maintain rhythmicity which is relatively coordinated with the exogenous cycle (Fig. 3C). Under these circumstances the rhythmicity cannot be the output of an hour glass mechanism.

When the period of the MAP/H_2O cycle is extended to 48h, rhythmicity persists with a much shortened period (Fig. 3E). This result is also inconsistent with the hour glass model; however it is not clear why the period shortens. The effect may be related to the effect of a single withdrawal of MAP for 8 h which produces a highly variable phase advance (Fig. 3A) instead of the delay predicted by the hour glass model.

Finally, MAP-induced locomotor rhythmicity sometimes persists for a week or more after MAP is completely withdrawn. Such rhythmicity persists in only about 53% of the animals and is usually sloppy, with a short period (Fig. 3D). These results suggest that MASCO is either heavily damped or weakly coupled to locomotor activity.

If we accept that MASCO is a *bona fide* circadian oscillator, physically separate and distinct from the SCN, the behavior of intact mice exposed to MAP

and the sex and strain differences that we have observed are understandable as interactions between two oscillators, SCN and MASCO. Intact mice exposed to MAP often generate rhythms with two components. The shorter of these disappears when the SCN is lesioned or suppressed with LL and is therefore likely to be the result of rhythmic SCN output. The longer period component persists after SCN lesion and therefore must reflect rhythmicity of MASCO. In C57BL/6 mice these two components interact and influence each other (Fig 1A) and so they must be coupled. Note that although the two components interact, MAP does not rescue the SCN component in LL-treated mice which have a normal, though suppressed, SCN (Fig. 1C).

Intact male C3H mice rarely show a second, long component, and when they do, the two components do not appear to interact (Fig. 2A). Female C3H mice never show a second component, although, like the males, they increase α in response to MAP. Both sexes express robust MASCO-driven activity when the SCN is lesioned or suppressed with LL (Fig. 2 B & C). It seems likely that both the difference between C57BL/6 and C3H mice and the differences between C3H males and females are due to differences in the strength of coupling between SCN and MASCO. Specifically, coupling from SCN to MASCO may be relatively stronger in C3H than in C57BL/6 mice, and stronger in C3H females than in males. The periods of MAP-induced rhythms are considerably shorter in both SCN-lesioned and LL-treated C3H mice than in their C57BL/6 counterparts.

The period of MASCO is thus shorter and therefore closer to the period of the SCN in C3H than in C57BL/6 mice, perhaps enabling stronger coupling.

MASCO and the food-entrainable oscillator (FEO) are two examples of extra-SCN circadian oscillators that contribute to behavioral rhythmicity. Although there is presently no evidence either way, it has been suggested that the two rhythmic behaviors are driven by the same physical oscillator (Honma, Honma et al. 1988; Honma, Honma et al. 1989; Hiroshige, Honma et al. 1991; Honma, Kanematsu et al. 1992; Davidson, Tataroglu et al. 2005). Because MAP is an addictive drug which acts primarily on dopaminergic cells in the brain, it is tempting to speculate that by exposing animals to chronic MAP we have somehow tapped into a connection between the circadian and the reward systems (for discussion see (Davidson, Tataroglu et al. 2005). There are other, independent suggestions that such a connection exists (Abarca, Albrecht et al. 2002; Ralph, Ko et al. 2002; McClung, Sidiropoulou et al. 2005), and if it does, it is likely to prove important in the understanding of reward and the treatment of addiction.

Acknowledgements:

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Figure 2-1 Wheel running activity of C57BL/6 mice treated with MAP

Chronic MAP was administered for the duration and dose indicated by the arrow on the left of the actogram. **A.** Intact mice were kept in 12/12 LD for 4 days and then released to DD. MAP significantly increased the total daily activity, duration of activity (alpha) and the period length within 8 days. 33% of the mice showed two rhtyhmic components which showed relative coordination. All effects disappeared after withdrawal. **B.** MAP induced robust rhythms in SCN-lesioned mice in DD. **C.** Similar rhythms were also induced by MAP in LL-treated arrhythmic intact mice. Only one rhythmic component was observed in either SCN-lesioned or LL-treated arrhtyhmic mice.

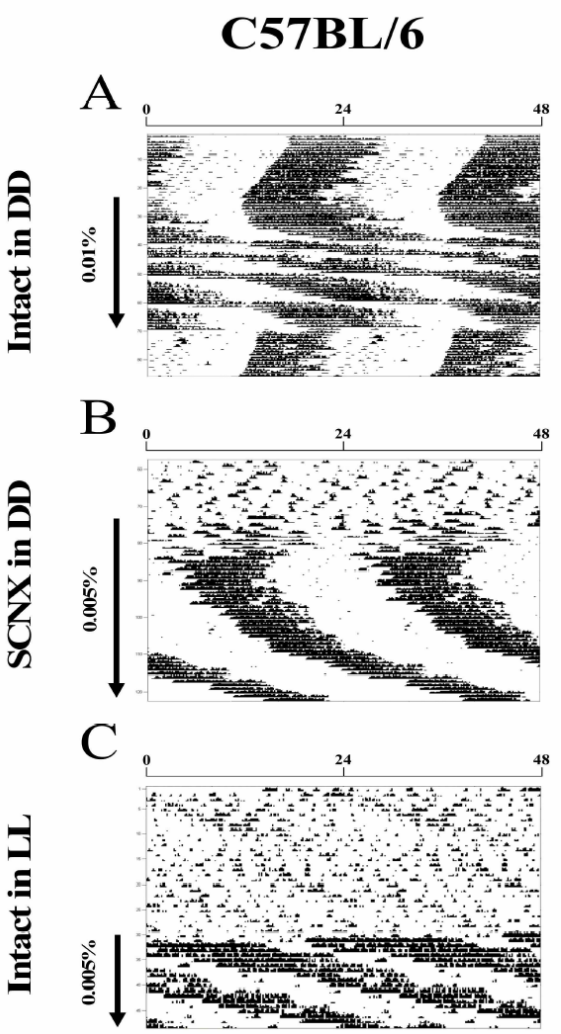

C57BL/6
A
0
24
48
Intact in DD
0.01%
B
0
24
48
SCNX in DD
0.005%
C
0
24
48
Intact in LL
0.005%

Figure 2-2 Wheel running activity of C3H mice treated with MAP

Chronic MAP was administered for the duration and dose indicated by the arrow on the left of the actogram. A. Intact C3H mice were kept in 12/12 LD for 4 days and then released to DD. MAP increased alpha in all C3H mice, but did not reliably induce a second component of the rhythm or lengthen the period as it did in the intact C57BL/6 mice. However, some male C3H mice exhibited a temporary 2nd component of the rhythm (best example is shown). This 2nd component was not observed in the females and disappeared after withdrawal in males. B. Effects of MAP on SCN-lesioned C3H mice were very similar to the effects on C57BL/6 mice C. LL-treated arrhythmic intact C3H mice also showed MAP-induced rhythms .

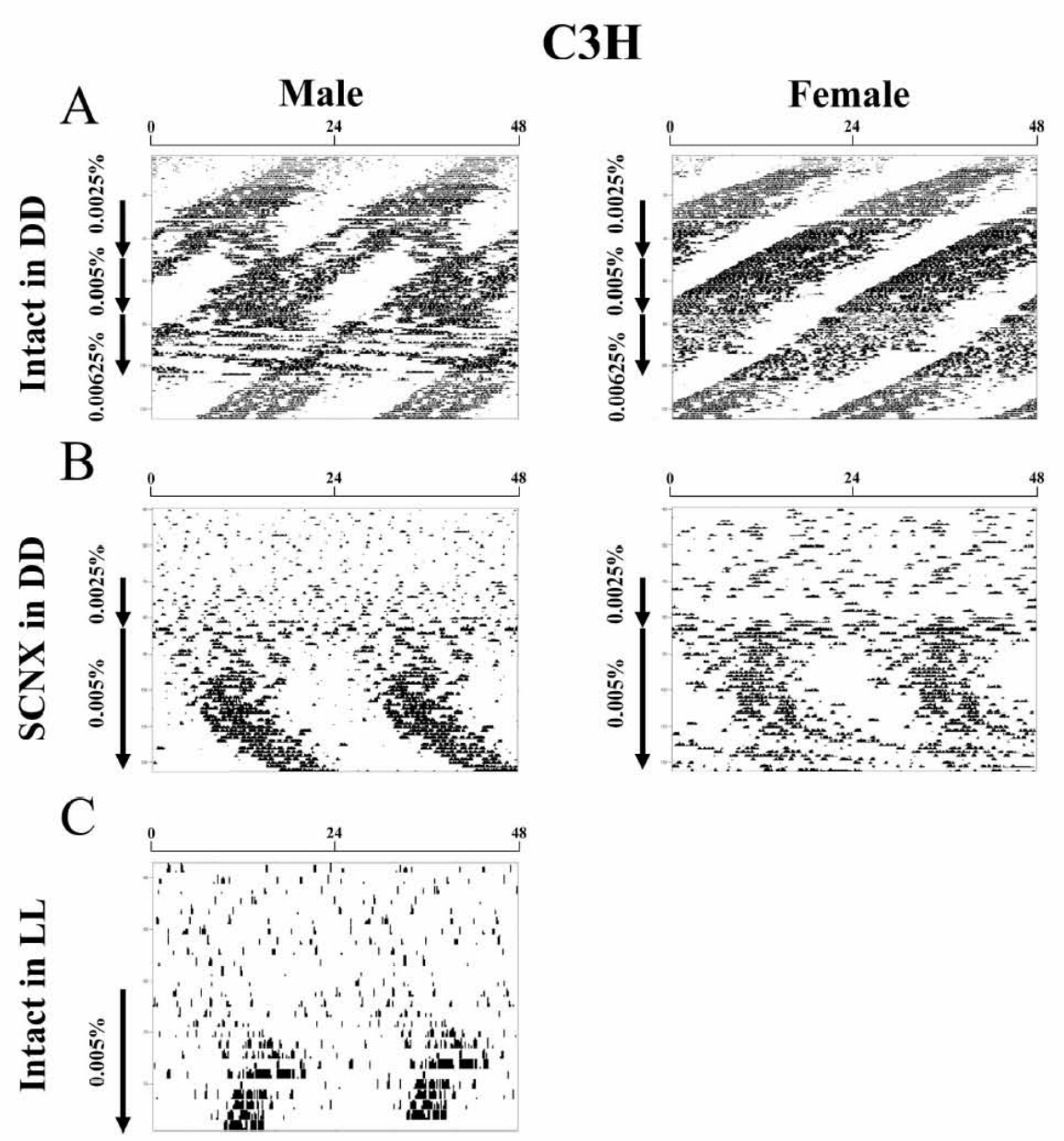

C3H
A
Male
Female
0
24
48
Intact in DD
0.0025%
0.005%
0.00625%
B
SCNX in DD
C
Intact in LL

Figure 2-3 Tests of the “hourglass” hypothesis

0.005% MAP was administered to SCNX arrhythmic C57BL/6 mice in DD and stable MAP-induced activity rhythms were confirmed by periodogram analysis. The portions of the actograms that contain the SCN-lesion, post-operative care and initial period of MAP administration are not shown (except in E). MAP was available at all times except those shown by shading when the mice received only water. **A.** Replacement of MAP with pure water for 8 hours at CT12 resulted in a phase-advance (Actogram is plotted at the period length of this mouse before the 8 hour withdrawal) **B.** MAP-induced rhythms entrained to the 14 hours of MAP/10 hours of water schedule with transients. **C.** MAP-induced rhythms showed relative coordination during the shorter period 14/8 MAP/water schedule (Actogram is plotted at 22 hours to improve visualization of possible entrainment). **D.** MAP-induced rhythms occasionally persisted after withdrawal for up to two weeks. **E.** MAP-induced rhythms persisted for longer durations when MAP was alternated with water every day (24/24 MAP/water schedule. Note that rhythmicity persists even on days when only water was available. The entire duration of the schedule has been shaded for visual clarity.

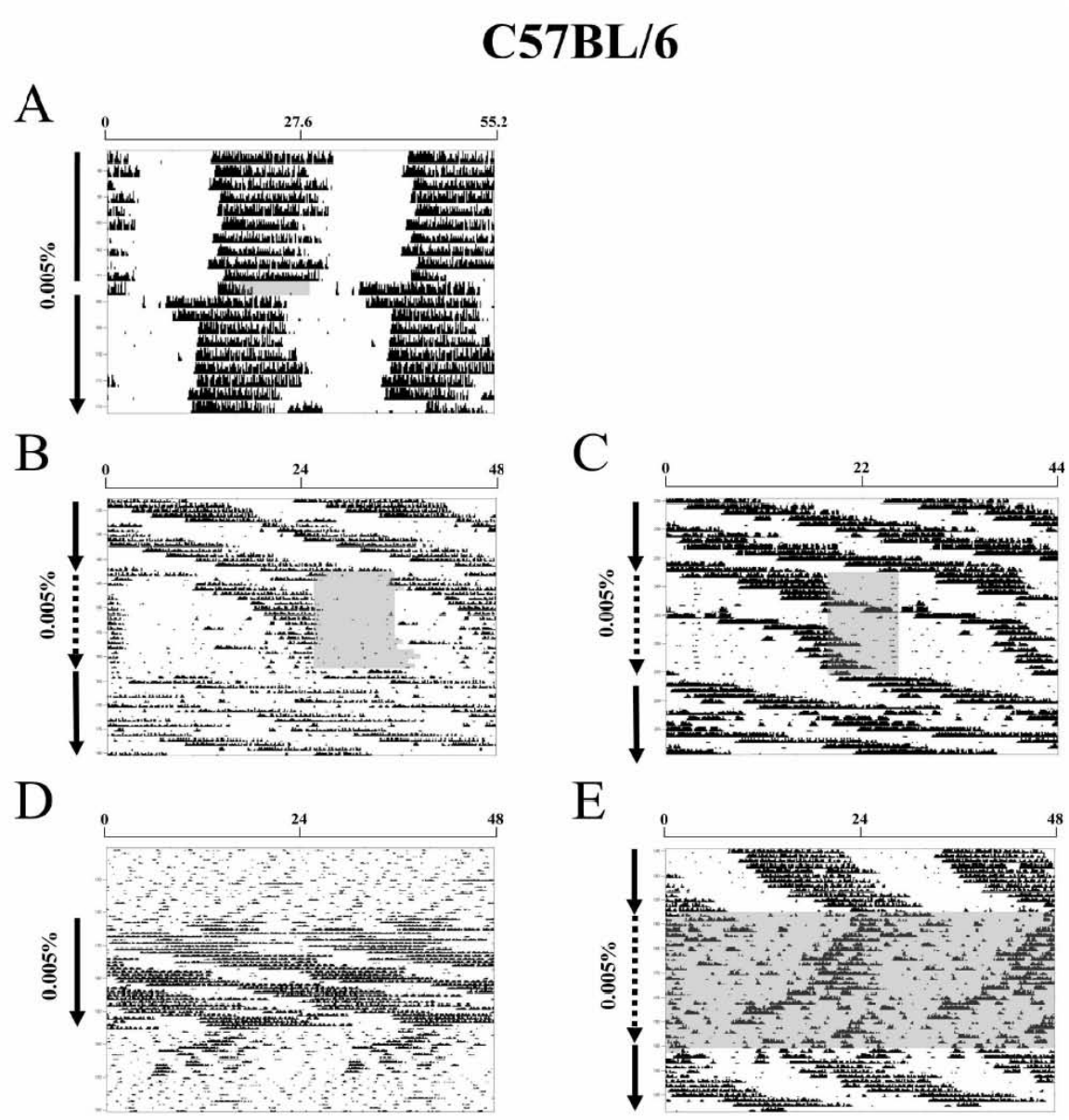

C57BL/6
A
0
27.6
55.2
0.005%
B
0
24
48
0.005%
C
0
22
44
0.005%
D
0
24
48
0.005%
E
0
24
48
0.005%

Table 2-1 Effects of methamphetamine on the period length

Intact mice are separated into two groups; mice that showed a single rhythmic component and mice that showed two rhythmic components during methamphetamine treatment. Sex differences were only observed in the intact C3H mice (see text). LL-treated mice are divided into two groups; mice that stayed rhythmic in LL and mice that became arrhythmic in LL. Numbers in parentheses indicate the number of mice. Periodogram analyses were performed in the range of 19 to 34 hours using the Clock lab analysis program (Actimetrics, Evanston, IL) on at least 10 days of continuous data. The block size was 10 minutes. Period lengths are shown as average ± standard deviation. Second components are shown as an additional average ± standard deviation (if n>2) whenever they were present.

Table 1. Effects of Methamphetamine on the Period Length

	Pretreatment	*0.0025% MAP*	*0.005% MAP*	*0.00625% MAP*	*0.01% MAP*	*Posttreatment*
Intact C57BL/6						
(0.005 → 0.01% MAP)						
Single component	23.64 ± 0.11 (15)		24.04 ± 0.21 (15)		24.17 ± 0.21 (15)	23.74 ± 0.32 (15)
Two components	23.75 ± 0.17 (4)		24.17 ± 0.30 (4) and 27.50		23.85 ± 0.03 (4) and 26.96 ± 1.51 (4)	23.25 ± 0.29 (4)
Intact C57BL/6						
(0.01% MAP only)						
Single component	23.62 ± 0.17 (13)				24.14 ± 0.13 (13)	23.71 ± 0.31 (13)
Two components	23.67 ± 0.16 (10)				24.04 ± 0.15 (10) and 27.53 ± 1.98 (10)	23.72 ± 0.26 (10)
Intact C3H						
Single component (males)	23.44 ± 0.10 (3)	23.44 ± 0.10 (3)	23.44 ± 0.10 (3)	23.44 ± 0.10 (3)		23.39 ± 0.10 (3)
Single component (females)	23.30 ± 0.18 (5)	23.47 ± 0.14 (5)	23.53 ± 0.14 (5)	23.70 ± 0.38 (5)		23.40 ± 0.09 (5)
Two components (males)	23.61 ± 0.10 (3)	23.50 ± 0.17 (3) and 29.50, 24.67	23.50 ± 0.17 (3) and 29.00	23.58 ± 0.12 (3) and 26.83		23.56 ± 0.10 (3)
SCNX C57BL/6						
Arrhythmic	Arrhythmic (23)		26.29 ± 1.53 (23)			23.54 ± 1.13 (8) or Arrhythmic (9)
SCNX C3H						
Arrhythmic	Arrhythmic (4)		24.54 ± 0.64 (4)			
LL-treated intact C57BL/6						
Arrhythmic	Arrhythmic (3)		27.44 ± 0.20 (3)			
Rhythmic	26.29 ± 1.53 (8)		28.21 ± 0.65 (8)			
LL-treated intact C3H						
Arrhythmic	Arrhythmic (3)		24.63 ± 1.27 (3)			
Rhythmic	25.22 ± 0.82 (3)		24.94 ± 0.59 (3)			

Chapter 3: Discussion

Discussion

The results of our experiments reported in chapter 2 clearly show that mice are a good model in which to study the effects of methamphetamine (MAP) which were previously published in rats(Honma, Honma et al. 1986). The differences such as more robust and clear activity rhythms, more pronounced second components of the rhythm and obvious sex and strain differences make the mice a better model. In addition, the currently available circadian mutant mice and other genetic tools further increase the value of mice in the study of the Methamphetamine-sensitive circadian oscillator (MASCO).

The results of the experiments in which we tested the hourglass explanation of the effects of MAP on circadian behavior strongly argue against such a model. Therefore, MASCO exists and is indeed a circadian oscillator. However, there are many interesting questions that were not answered by our studies; where is MASCO localized? Which known/unknown circadian genes are involved in its functioning? How is MASCO coupled to SCN or other oscillators in the body? Which neurotransmitter system is critical for MASCO? Does MASCO have any similarities with the Feeding-entrainable oscillator (FEO)? In this discussion, I will revisit such questions and speculate how we can approach them using our current knowledge.

The comparison between intact and SCN-lesioned (SCNX) C57BL/6 and C3H mice suggests that MASCO is more strongly coupled to SCN in the C3H strain. We do not know how this coupling is achieved. However, we can thoroughly study the differences between these two strains to elucidate the mechanism required for this coupling. For example, the C3H mice produce melatonin while the C57BL/6 mice don't. If melatonin is involved in this coupling, one such study would be to pinealectomize C3H mice and administer MAP and see whether we can achieve the same effects (or more pronounced effects) of MAP on wheel running behavior. An alternative approach would be to administer melatonin to C57BL/6 mice to test whether we can diminish the effects of MAP in this strain.

Administration of a high dose of MAP to intact C57BL/6 mice produces two components of the circadian behavior rhythm that show relative coordination to each other. The shorter period length component, which we previously named the "first component", disappears when SCN is lesioned or the intact mice were kept in constant light (LL). This suggests that the first component is representative of the effects of MAP on SCN. Our results show that the period length of the first component increases in intact mice during MAP administration. This may occur either by the actions of MAP directly on the SCN or by the effect of coupling between SCN and MASCO where MASCO "pulls" SCN towards a longer period length.

It is still unclear whether MAP has any direct effect on the SCN. A single injection of MAP does not induce *Per1, Per2* or *Per3* expression in the SCN while it induces the expression of these genes in areas such as Caudate-Putamen (CPU) in mice(Nikaido, Akiyama et al. 2001). Similarly, chronic MAP does not affect the *Per1* expression profile in the SCN of rats ex vivo (Masubuchi, Honma et al. 2000) or the firing activity of SCN neurons in vitro(Moriya, Fukushima et al. 1996). In contrast to these results, MAP infusions into the SCN of rats using microdialysis probes increases extracellular serotonin levels (Ozaki, Nakahara et al. 1991). In addition, MAP seems to activate the 5-HT1A receptors through increasing 5-HT levels in the rat SCN and inhibit light- induced phase shifts in hamsters (Moriya, Yamanouchi et al. 1996). However, species differences with respect to the effects of MAP may exist between rats, mice and hamsters.

It is also possible that the lengthening of the period of the first component is probably due to the coupling between SCN and MASCO in vivo. In fact, in vivo multiple-unit activity (MUA) recordings from hamsters during chronic MAP treatment shows that MUA follows the locomotor rhythm whose period length is increased by MAP administration(Omata and Kawamura 1988). Interestingly, our data from SCNX mice and intact mice show that the period length of the second component does not change when SCN is lesioned which suggests that SCN can not “pull” MASCO towards itself. However, the comparison between intact and SCNX C3H mice suggests that the presence of SCN inhibits MASCO or its output.

Although, involvement of dopamine, serotonin and noradrenaline in the effects MASCO were suggested(Honma and Honma 1995), it has never been fully tested. One way to address this question would be to apply repeated injections of high doses of MAP to deplete monoamines in the brain and then apply MAP chronically to test whether MAP induced increase in activity, alpha or period length can be observed. Our pilot experiments showed that such a regimen does not have any effect of these parameters. However, the data implied that the second component of the rhythm was less pronounced in the MAP treated mice. An alternative approach to test the involvement of dopamine would be to apply reagents such as 6-hydroxy dopamine (6-OHDA) or alpha-methly-paratyrosine which are known to cause depletions of dopamine in the brain.

Our experiments did not address the localization of MASCO. The search for the site for MASCO could prove to be as hard as the unsuccessful search for FEO. Nonetheless, it could be tested. Lesions of candidate brain areas can be performed and MAP can be administered to see whether the lack of those areas will inhibit or enhance the effects of MAP on circadian behavior. Alternatively, MAP can be applied to slice preparations from these areas in vitro or electrophysiological measurement can be made in vivo. It is important, however, to be careful in the interpretation of the results of these experiments. The lack of effects of MAP on circadian rhythms after such treatments could also mean that the suggested area is either involved in the MASCO itself or its output pathways.

We tried to elucidate the similarities between the molecular components of SCN and MASCO using several circadian mutant mice that were available to us (see appendix for results). However, it should be noted that these experiments can only serve as pilots since we did not have adequate number of mice in each strain to allow proper statistical analysis. In addition, we haven't replicated these experiments in SCNX circadian mutant mice which will allow better clarity in the actograms and differentiation of the observed rhythm from the SCN-related first component. Therefore, we cannot assume that our observations were truly reflections of the effect of the mutation on MASCO function. However, we can still speculate about possible involvement of these known circadian genes in the effects of MAP.

The *Npas2*^{tm1Slm} mutant mice did not show any significant differences in their response to MAP in terms of circadian behavior (Figure A-1). The next step in this experiment is to create a *Clock*^{m1Jt} */ Npas2*^{tm1Slm} double mutant mouse line and test the effects of MAP since it was suggested that CLOCK and NPAS2 can substitute for each other in the circadian system(Reick, Garcia et al. 2001).

The results from *Cry1* -/- *Cry2* -/- double mutant mice were particularly interesting (Figure A-2). MAP was able to induce robust rhythms in the *Cry1* -/- *Cry2* -/- double mutant mice. Although we were able to test only 2 double mutant mice, similar effects were also observed by the Honma group in Japan (unpublished). Our data suggests that *Cry1* mutation (or *Cry1* mutation in combination with *Cry2*

mutation) has a significant effect on the latency of the effects of MAP on the circadian rhythm. *Cry1* homozygous mice showed the MAP-induced rhythms much later than their heterozygous counterparts. This suggests that *Cry1* is not involved in the final rhythm induced by MAP, but in its development. One explanation for this could be involvement of *Cry1* in the coupling between SCN and MASCO where *Cry1* (and/or *Cry2*) decreases the inhibition of MASCO by the presence of SCN. This can hypothesis be tested in SCNX *Cry* mutants. If the assumption is true, then the effects of MAP should be observed much earlier in the *Cry1* -/- *Cry2* -/- double mutant mice.

Similar to *Cry* mutation(s), *Per1* mutation also seems to change the latency of the effects of MAP on circadian behavior (Figure A-4). However, both *Per1* and *Per2* do not seem to be involved in the core oscillation of MASCO since robust MAP induced rhythms were observed in both groups of mice (Figure A-5a and b). Unfortunately, we can not interpret the results of our results from the *Per3* knock-out mice, because we were not able to run proper 129/sv background controls (Figure A-6). The lack of effect of MAP in these mice might be due to this background which is well known to have diminished responses to rewarding drugs such as cocaine(Thomsen and Caine 2006).

The results of our experiments with circadian mutant mice under MAP treatment are not adequate to propose a list of known genes that are involved in MASCO function. However, it is important to follow up these results and determine the

differences between the molecular components of SCN and MASCO. These studies can also provide us with new molecular markers for localizing MASCO. For example, if NPAS2 seems to be critical for MASCO functioning but not CLOCK then it is likely that MASCO will be localized in the forebrain where NPAS2 is expressed(Reick, Garcia et al. 2001). In addition, if some of the known circadian genes are found out to be involved in MASCO, but not all, then interactions of the protein products of these genes might reveal previously unknown circadian genes which will increase our knowledge of the molecular basis circadian rhythms. Furthermore, some of these interacting genes might already be known to be involved in processes such as reward or feeding, for example, which can elucidate the connections of MASCO with these systems and its natural role in the body.

The first and foremost aim of the future studies of MASCO should be identification of the molecular components of this oscillator and its localization which will provide the necessary background for future studies. Utilization of the mouse strains presented in this thesis will provide an easy to obtain standard model where MASCO can be studied using latest developments in genetics and molecular biology. Identification of the natural role of MASCO in the body and its interactions with other oscillators in the body is of both scientific and clinical significance and will greatly improve our understanding of the circadian system in mammals.

Appendix: Molecular Components of the Methamphetamine-Sensitive Circadian Oscillator (MASCO)

Introduction:

Molecular components of MASCO are unknown. However, it's known that effects of MAP on the circadian behavior are still present in $Clock^{m1Jt}$ mutant mice (Masubuchi, Honma et al. 2001). In this experiment, authors administered 0.005% MAP in drinking water and observed MAP-induced rhythms in these arrhythmic $Clock^{m1Jt}$ mutant mice in DD. The rhythm disappeared after withdrawal within days in most mice, but in some of the animals, it persisted for several weeks with period lengths ranging from 26.8 to 29.3 hours. It should be noted that the period length of the persisting rhythm in our SCNX mice have always been less than 24 hours. Other concerns about this experiment were previously mentioned in the molecular components of MASCO in the Introduction section of this thesis. In summary, NPAS2 is known to be a functional substitute for CLOCK and it is possible that MASCO might utilize either or both of these proteins; hence may still be functional in the absence of CLOCK. In addition, since the mice used in this experiment are intact, it is possible that the observed rhythms in these mice during MAP administration may result from an effect of MAP on the SCN and represent the first component of the rhythm observed in wild-type mice. Furthermore, the $Clock^{m1Jt}$ mutant mice still possesses a CLOCK protein (although mutated) and it is possible that MASCO may not use the mutated region of this protein.

We applied the same dose of (%0.005) MAP to *Npas2*^{tm1Slm} , *Per1*^{ldc} , *Per2*^{ldc} and *Per3* mutant mice as well as *Cry1* -/- *Cry2* -/- double mutant mice and measured their wheel running activity in DD. There was no significant difference in the distribution of ages throughout the experiments. The following figures represent the variety of effects we observed in these mice during the MAP treatment.

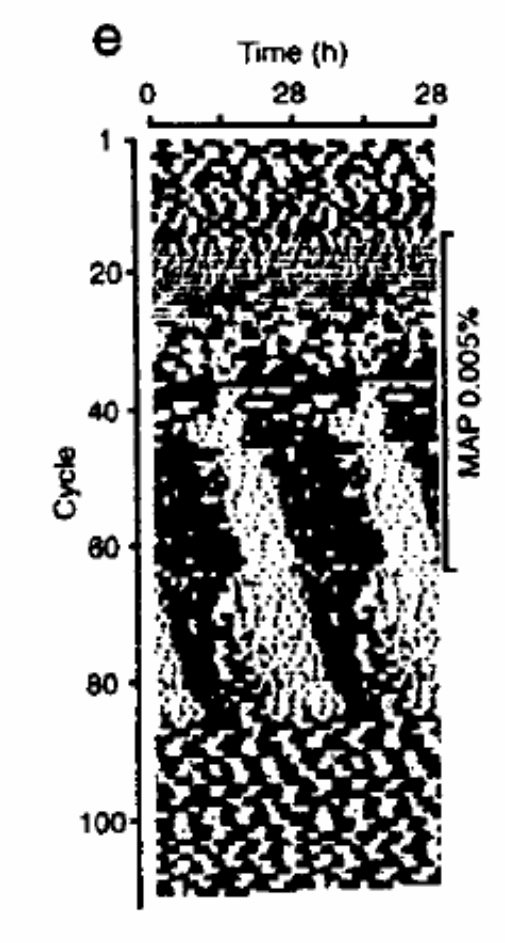

e
Time (h)
0
28
28
1
20
40
60
80
100
Cycle
MAP 0.005%

Figure A-1 Effects of MAP in Clock^{m1Jt} mutant mice

In this experiment, homozygous intact Clock^{m1Jt} mutant mice (on congenic C57BL/6 background) were kept in DD. After confirmation of arrhythmicity, they were administered 0.005% MAP in drinking water. Within 30 days, MAP-induced activity rhythms were observed. The rhythms persisted for up to 3 weeks after withdrawal. The period lengths of these rhythms were between 27.5 to 42.9 hours during MAP treatment and 26.8 to 29.3 hours after withdrawal. All effects of MAP disappeared after this period and the mice became arrhythmic again. It should be noted that the period length of persisting rhythms in our SCNX experiments were always shorter than 24 hours and lasted up to 14 days after withdrawal. Figure reproduced from (Masubuchi, Honma et al. 2001). Chronic MAP was administered for the duration and dose indicated on the right of the actogram.

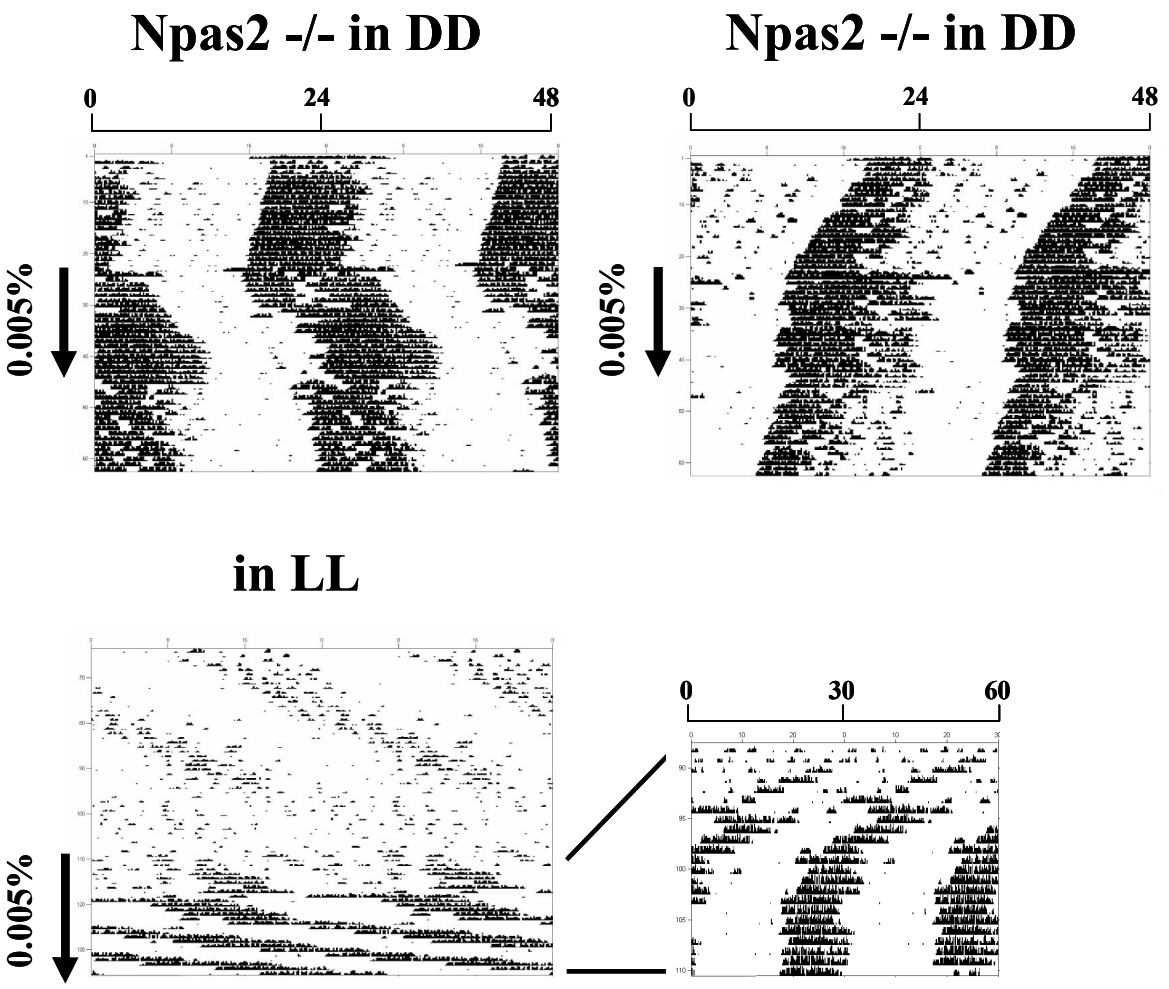

Npas2 -/- in DD
0
24
48
0.005%
Npas2 -/- in DD
0
24
48
0.005%
in LL
0.005%
0
30
60

Figure A-2 Effects of MAP in *Npas2*^{tm1Slm} mutant mice

Homozygous intact *Npas2*^{tm1Slm} mutant mice (n=3, on congenic C57BL/6 background) were kept in DD and wheel running behavior was recorded. After confirmation of the pretreatment period length, 0.005% MAP was administered in drinking water. MAP increased the activity, alpha and period length in all 3 mice within days. The effects disappeared almost immediately after withdrawal. The effects of MAP on these 3 *Npas2*^{tm1Slm} mutant mice and their 7 heterozygous and 2 wild-type controls (data not shown) were similar to C57BL/6 mice we tested in chapter 2. We did not observe any second components in any of the *Npas2*^{tm1Slm} mice. However, we did not record their activity for as long as we recorded from C57BL/6 mice that were mentioned. When we released these mice to constant light (LL), all 3 *Npas2*^{tm1Slm} knock-out mice became arrhythmic and showed robust MAP-induced rhythms with period lengths of 26.44, 26.83 and 29.45 hours. We did not observe second components in LL, similar to the case in intact C57BL/6 mice in LL.

Cry 1 -/- Cry2 -/- in DD

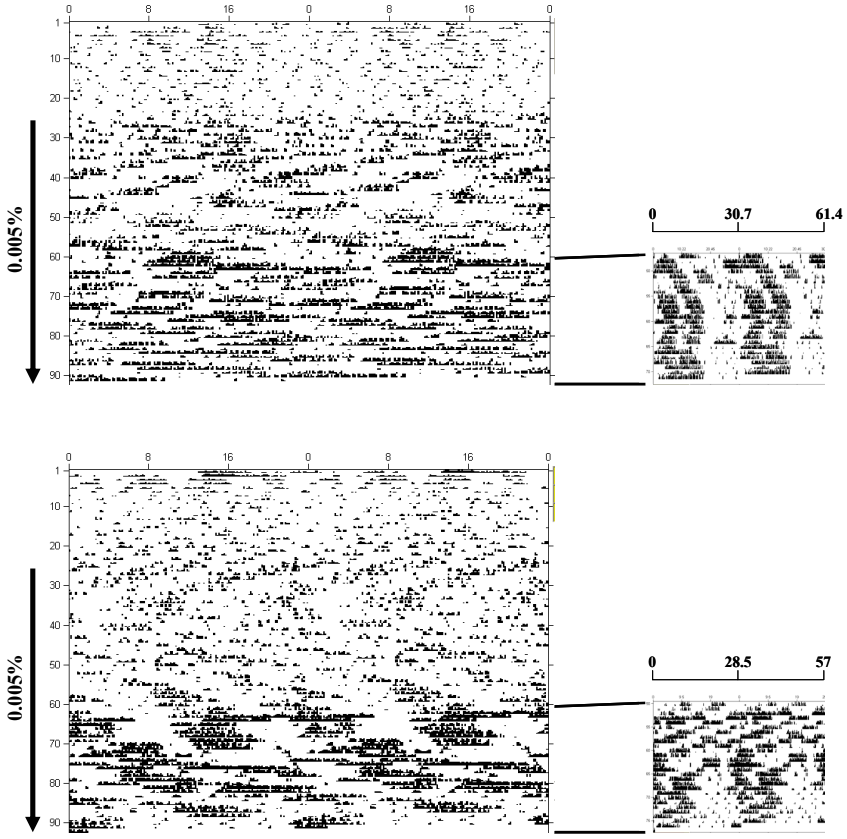
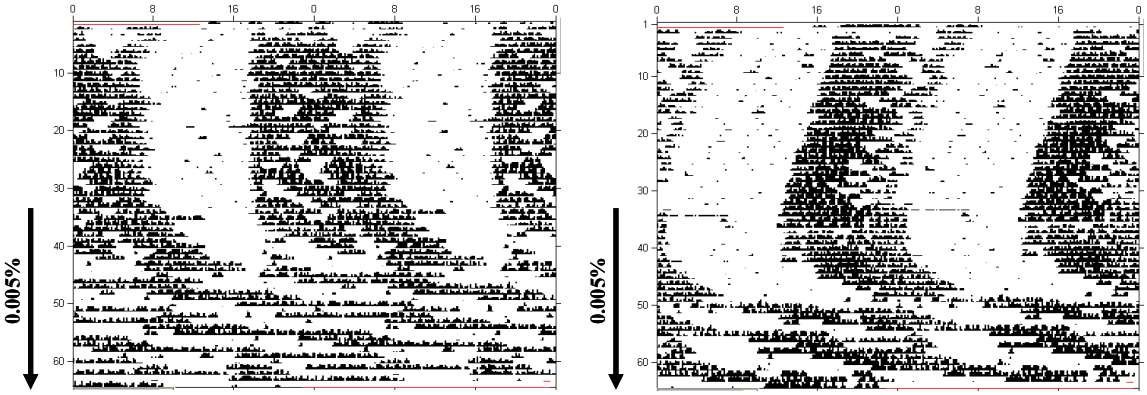


Figure A-3 Effects of MAP in *Cry1* -/- *Cry2* -/- double mutant mice

Homozygous intact *Cry1* -/- *Cry2* -/- double mutant mice (n=2, on congenic C57BL/6 background) were kept in DD and wheel running behavior was recorded. After confirmation of arrhythmicity, 0.005% MAP was administered. Both mice showed robust MAP-induced rhythms with period lengths of 28.5 and 30.67 hours which is similar to what is observed in SCNX C57BL/6 mice treated with the same dose of MAP. However, there was a significantly longer transient period before these rhythms were observed compared to wild-type C57BL/6 mice treated with MAP. We also used *Cry1* +/- *Cry2* -/- mice alongside these double mutant mice. Interestingly, they also showed similar effects of MAP, but without the significantly long transient period. The effects in these mice were observed within two weeks which is similar to SCNX wild-type C57BL/6 mice.

Per1 +/- in DD



Per1 -/- in DD

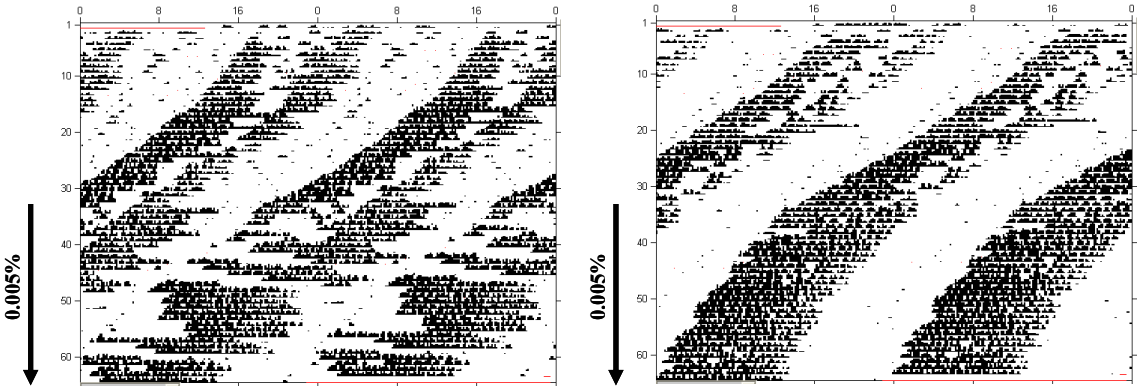


Figure A-4 Effects of MAP in *Per1*^{ldc} mutant mice

Homozygous (n=2) and heterozygous (n=2) intact *Per1*^{ldc} mutant mice on congenic C57BL/6 background were kept in DD and wheel running behavior was recorded. After confirmation of the pretreatment period length, 0.005% MAP was administered. MAP increased the alpha and the period length in both groups within days, similar to intact wild-type C57BL/6 mice. In both groups, one of the mice showed a second component of the rhythm (right actogram in heterozygous group and left actogram in the homozygous mutant *Per1*^{ldc} mice). The period length of the second component was similar to wild-type C57BL/6 mice in both groups. However, they were more pronounced in the heterozygous group.

Per2 -/- in DD

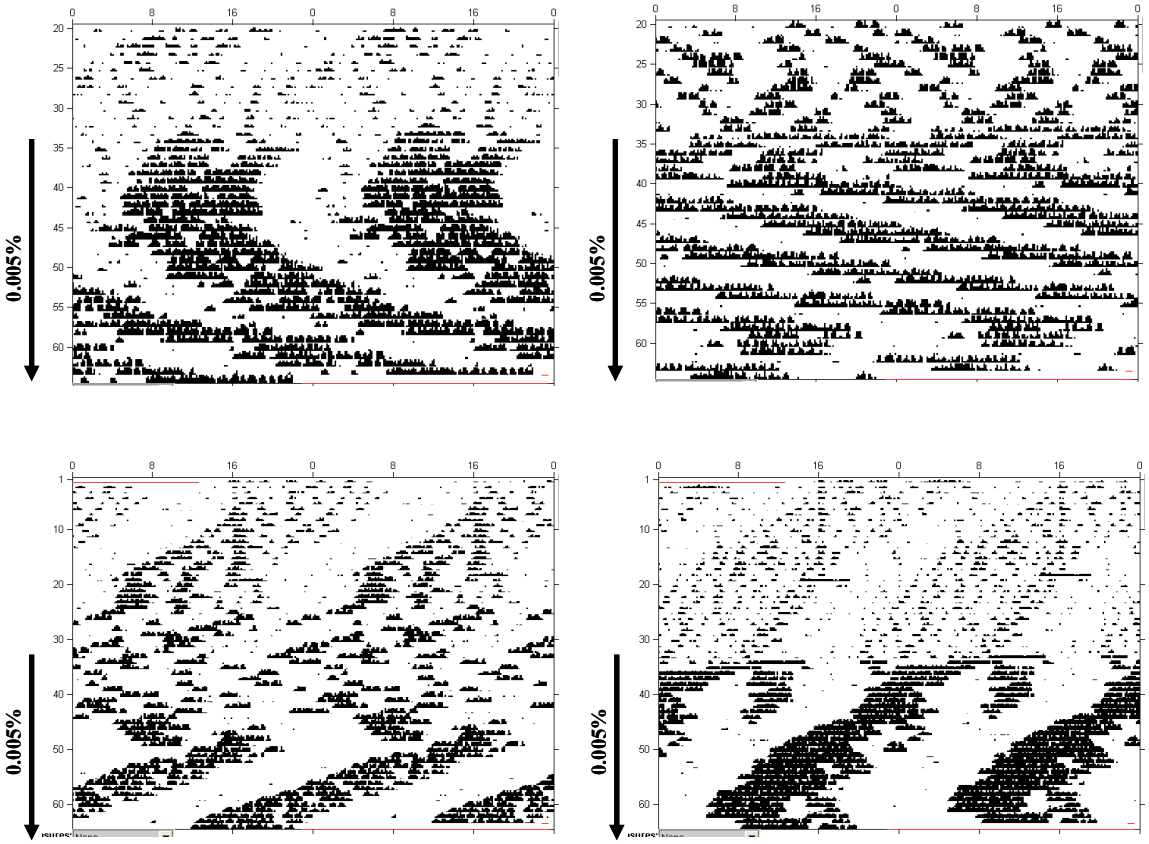


Figure A-5a Effects of MAP in *Per2*^{ldc} mutant mice

Homozygous (n=8) intact *Per2*^{ldc} mutant mice on congenic C57BL/6 background were kept in DD and wheel running behavior was recorded. 3 of 8 mice became arrhythmic in DD (top row). After confirmation of the arrhythmicity, 0.005% MAP was given to all mice. Effects of MAP in *Per2*^{ldc} mutant mice were variable. MAP did not have a significant effect on the period length of the mice that did not become arrhythmic (bottom row), but it did induce robust rhythms in the 3 mice they became arrhythmic in DD. The period lengths of the MAP-induced rhythm in the latter group were similar to SCNX wild-type C57BL/6 mice.

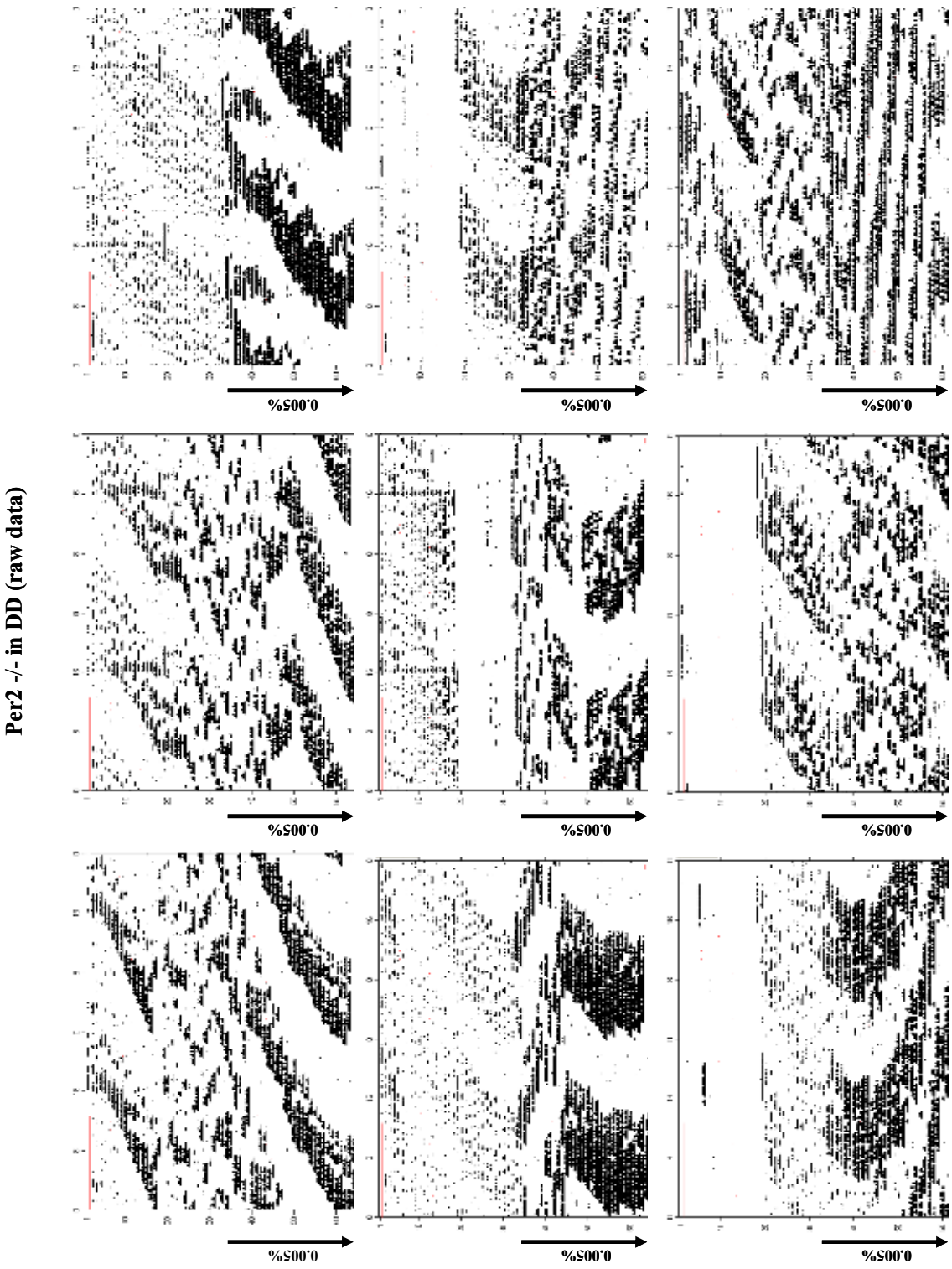

Per2 -/- in DD (raw data)
0.005%
0.005%
0.005%
0.005%
0.005%
0.005%
0.005%
0.005%
0.005%

Figure A-5b Effects of MAP in *Per2*^{ldc} mutant mice (raw data)

All actograms from this experiment are provided to illustrate the variable effects of MAP on the circadian rhythms of *Per2*^{ldc} mutant mice.

Per3 -/- in DD

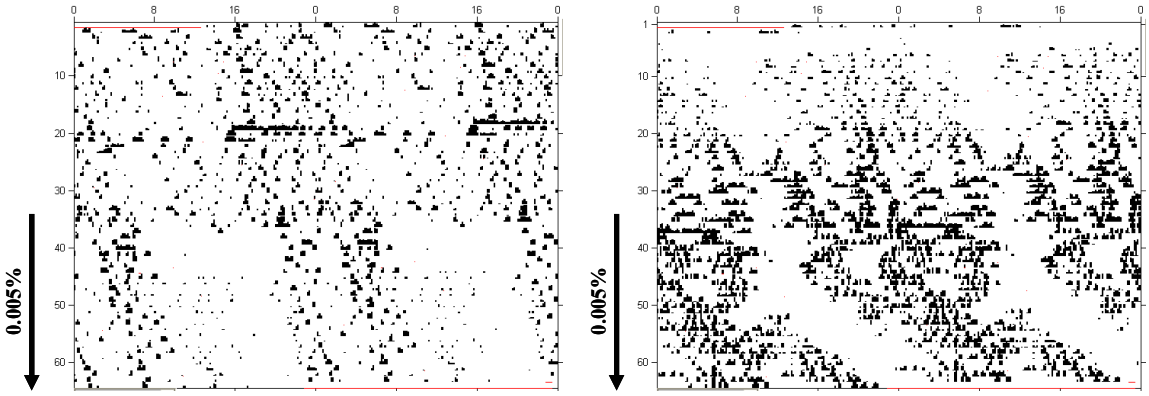


Figure A-6 Effects of MAP in *Per3* mutant mice

Homozygous (n=7) intact *Per3* mutant mice on 129/sv background were kept in DD and wheel running behavior was recorded. 2 of 7 mice became arrhythmic in DD (data not shown). After confirmation of the arrhythmicity or the pretreatment period length, 0.005% MAP was given to all mice. MAP failed to significantly increase the period length of the mice that were rhythmic before the treatment. The largest increase was observed in the right-hand actogram shown. Although MAP induced rhythmicity in the arrhythmic mice, the period length of the induced rhythm was similar to the mice that were rhythmic before the treatment. We did not have the background controls for these mice.

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