MASKING: THE ACUTE EFFECTS OF LIGHT ON THE BRAIN AND BEHAVIOR

By

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ABSTRACT

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Masking of behavior by external stimuli works with the circadian system to ensure that animals are active during the correct time-of-day. Light for diurnal and nocturnal species produces different masking responses, enhancing activity for diurnal species and suppressing activity for nocturnal species. Few studies have examined the neural mechanisms of masking; none these experiments use animals active during the day. The first experiment of this dissertation uses the protein of the immediate-early gene cFOS to compare activation of brain regions to light between nocturnal mice and diurnal grass rats during a time-point where they showed a distinct behavioral dichotomy in response. Grass rats showed a consistent increase in activation in areas that receive retinal innervation or were related to sleep/arousal, while mice showed either no difference or a decrease in activation with the exception of the SCN. This study demonstrates the differences in behavioral and neurological responses to masking pulse of light between a nocturnal and diurnal rodent species.

We next examined the functional role of the ventral subparaventricular zone (vSPZ) on masking to light in the grass rat. The vSPZ uniquely responded to light in diurnal grass rats, receives direct retinal innervation from the eye and after ablation showed a possible alteration in masking. Schwartz et al. (2009)

showed that grass rats with damage to the vSPZ had bouts of arrhythmia in LD conditions, indicating that the masking system may be altered. In Chapter 3, we tested the functional for of the region by bilateral lesioning the vSPZ and directly testing the effects of light on masking. Animals with complete ablations still increased activity in response to light in two different masking protocols. In Chapter 4, we examined another brain structure that could have played a role in masking, the pineal gland. Melatonin is a hormone produced by the pineal gland that plays a role in circadian rhythms and seasonality. The expression of melatonin is sensitive to light exposure, additionally, the presence of the hormone also feedbacks and influences the effects of light on the brain. Additionally, pinealectomy in rats alters the behavior profile of activity in LD conditions. Removal of the pineal gland in the grass rats did not influence the ability of animals to mask to light or the animal's circadian rhythm of activity.

In summary, the work presented in this dissertation demonstrates the immediately impact of light on behavior and the possible brain regions that may play a functional role in the masking response. We established that two neural structures when ablated or removed do not impact the grass rat's ability to respond to light with an increase in activity. Here we broaden the scope of research into the neural mechanisms of masking to include a diurnal species, beginning the journey toward understanding the neural changes needed for a species to transition between temporal niches.

This dissertation is dedicated to my family whose love and support inspired me to pursue my scientific dreams; and to my advisor, Dr. Laura Smale for allowing me to accomplish my goals with her invaluable guidance and encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

Abbreviation	Definition
ABC	Avidin-Biotin Peroxidase Complex
AHA	Anterior Hypothalamic Area
ANOVA	Analysis of Variance
BT	Body Temperature
CT	Circadian Time
CTβ	Cholera Toxin Beta-subunit
DAB	3,3'-diaminobenzidine
DD	Constant Dark Conditions
DLG	Dorsal Lateral Geniculate
DMH	Dorsomedial Hypothalamic Nucleus
DP	Dark Pulse
DR	Dorsal Raphe
GA	General Activity
GHT	Geniculohypothalamic Tract
HR	Heart Rate
IACUC	Institutional Animal Care and Use Committee
ICC	Immunocytochemistry
IGL	Intergeniculate Leaflet
IML	Intermediolateral Nucleus

ipRGC ……..melanopsin-expressing retinal ganglion cells

IRs……..Infrared Motion Detectors

LC ……..Locus Coeruleus

LD ……..Light:Dark Cycle

LH ……..Lateral Hypothalamus

LHb ……Lateral Habenula

LP ……..Light Pulse

LL ……..Constant Light Conditions

NMA……..N-Methyl-DL-aspartic acid

NMDA……N-Methyl-D-aspartic acid

NPY……..Neuropeptide Y

PBS……..….Phosphate Buffered Saline

PINX……..Pinealectomy

PVN ……..Paraventricular Nucleus of the Hypothalamus

PVT……Paraventricuar thalamus

OC ……...Optic Chiasm

OD……Optical Density

OPN ……..Olivary Pretectal Nucleus

opt……..Optic Tract

RCh……Retrochiasmatic Area

REM ……..Rapid Eye Movement Sleep

RT-PCR……Real Time-Polymerase Chain Reaction

SCG ……..Superior Cervical Ganglion

SCN ……..Suprachiasmatic Nucleus of the Hypothalamus

SCNx …….Suprachiasmatic Nucleus Lesions

VLPO ……...Ventrolateral Preoptic Nucleus

VMH……..Ventromedial Hypothalamic Nucleus

vSPZ …….Ventral Subparaventricular Zone

SPZx…… Ventral Subparaventricular Zone Lesion

ZT……....Zeitgeber Time

CHAPTER 1

Introduction

General Introduction

The cyclic pattern of the earth's rotation has created systematic and predictable daily rhythms in a range of environmental variables. Changes in the amount of ambient light and in temperature across the day produce unique patterns of resources available at specific phases of the day-night cycle; this variation had led organisms to become specialized for specific temporal niches (Kronfeld-Schor & Dayan, 2008). At the two extremes of this adaptive spectrum are (1) animals that are active primarily during the day, (diurnal animals), and (2) animals active at night, (nocturnal animals; Refinetti, 2008). Species that occupy these two temporal niches have developed adaptations that optimize functioning in their respective environments. Most notably, in vertebrates the visual system differs greatly between diurnal and nocturnal species (Peichl, 2005). The relative number of their photoreceptors that are cones, and the variety of types of cones, are greater in diurnal than nocturnal animals, which allows them to use in the daylight for color vision (Ahnelt & Kolb, 2000). Additionally, regions of the brain devoted to vision are larger in animals that are active during the day than those most active at night (Campi & Krubitzer, 2010; Gaillard, Karten, & Sauve, 2013). Both of these adaptations presumably improve survival and reproduction by allowing animals to more effectively locate food and identify potential dangers (Daan, 1981). The evolutionary transitions between nocturnal and diurnal species have, therefore, required many changes within the body, including the brain.

The expression of diurnal and nocturnal activity patterns is regulated by two distinct mechanisms, masking and circadian timekeeping systems. It has been hypothesized that these systems first evolved because single-cell organisms were reliant on the sun for the production of energy through photosynthesis, and cells that were able to restrict this process to the day, wasted less energy and had an adaptive advantage. Simons (2009) suggests that before circadian systems evolved, organisms developed the ability to respond directly and immediately to the presence of the sun, a masking response. Eventually, such organisms acquired the cellular machinery needed to maintain an endogenous circadian timekeeping mechanism that enabled them to anticipate and prepare for daily changes in the photic environment. These systems are also present in multicellular organisms but have become more complex, such that, for example, in animals there are neural mechanisms that regulate behavior in a time-of-day dependant manner. Species that switch their temporal niche undergo changes in both circadian systems and masking mechanisms to properly adapt to a new temporal environment.

When mammals evolved from reptiles they moved from a diurnal to a nocturnal niche, but there have been multiple, parallel evolutionary pathways back to diurnality (Smale, Lee, & Nunez, 2003). Many studies have examined the differences between the circadian systems of nocturnal and diurnal rodents, and these have found numerous similarities in the central circadian pacemaker of mammals, the suprachiasmatic nucleus (SCN), and many differences in rhythms in brain regions beyond the SCN (Smale, Nunez, & Schwartz, 2008). Little, however, is known about the neural changes that occurred at these transitions to alter masking behavior.

In this introductory chapter, I will first provide an overview of masking, with a focus on the differences observed in nocturnal and diurnal species. I will define the terms associated with masking and provide examples of niche specific masking responses. I will provide an overview of current research into the neural mechanisms of masking, which has focused almost exclusively on nocturnal rodents. This work has been aimed at identification of the retinal cells that are components of the input system pivotal to the expression of masking, as well as brain regions that are innervated by these cells. Here, I will also review papers that describe studies of effects of lesions of particular brain regions on masking. Finally, I will outline the research questions that are addressed in chapters two, three and four.

Masking Definitions

Aschoff (1960) first described masking in terms of how it affects circadian rhythms. He deemed environmental factors that obscure the observation of the endogenous rhythm as masking factors, as they directly influenced the variable being measured and masked the influence of the pacemaker (Aschoff, Daan, & Honma, 1982). The masking response can, therefore, be thought of as an immediate behavioral or physiological response to the presentation of an environmental stimulus, and the mechanism that produce it can be broken down into three components: (1) the input system that signals the presentation of the masking stimulus, (2) neural mechanisms that process the signal, and (3) the behavioral or physiological endpoint. More recently, researchers have proposed that the masking response helps animals specialize for their temporal niche (Mrosovsky, Foster, & Salmon, 1999). Masking enhances or represses

the expression of the endogenous rhythm and allows animals to adjust their behavior in response to small but important changes in the environment, providing animals more flexibility in the expression of their rhythms (Marques & Waterhouse, 1994).

Many different environmental stimuli can function as masking agents, such as temperature (Hoffman, 1969), social cues (Aschoff & Vongoetz, 1988) and, most importantly, light (Aschoff & Vongoetz, 1989). I will focus the remainder of the review below on light-induced masking. There are also both behavioral and physiological variables that respond in an immediate manner to changes in the photic environment. Light, for example, immediately suppresses the secretion of endogenous melatonin at night in all animals with functioning pineal glands (Challet, 2007). Light also impacts the rest-activity cycle of animals but the direction of the response is dependent on the temporal niche an animal occupies (Redlin, 2001). Among day-active organisms, such as diurnal birds (Aschoff & Vongoetz, 1989), rodents (Shuboni, Cramm, Yan, Nunez, & Smale, 2012), and primates (Gander & Mooreede, 1983), light increases the level of activity, a process referred to as positive masking. Among nocturnal rodents (Butler & Silver, 2011; Mrosovsky et al., 1999; Pendergast & Yamazaki, 2011; Redlin & Mrosovsky, 1999b) and primates (Erkert, Gburek, & Scheideler, 2006), light decreases activity, a processes referred to as negative masking. Additionally, exposure to light increases sleep in nocturnal species, such as mice (Morin & Studholme, 2009; Studholme, Gompf, & Morin, 2013) and decreases it in diurnal ones, such as humans (Cajochen, 2007; Lockley et al., 2006; Teixeira et al., 2013). These responses to light help restrict animals to the temporal niche to which they are optimally adapted, and

masking responses become a major barrier standing in the way of evolutionary transitions between nocturnality and diurnality (Kronfeld-Schor & Dayan, 2008).

In the wild, different selective pressures, including the introduction of predators (Fenn & Macdonald, 1995), competition (Harrington et al., 2009; Shkolnik, 1971) and anthropogenic disturbances (Rasmussen & Macdonald, 2012), induce temporal niche switches in species. One nice illustration of how masking is altered by the presence of competition comes from studies of two species of spiny mice whose ranges overlap in the vicinity of the Dead Sea and that have partitioned their resources by occupying different temporal niches (Shkolnik, 1971). Masking behavior of the diurnal golden spiny mouse is different from that of the nocturnal common spiny mouse but it is not simply reversed. Rather, the golden spiny mouse has a positive masking response to daytime dark exposure and does not respond to nighttime light (Cohen, Smale, & Kronfeld-Schor, 2010; Rotics, Dayan, Levy, & Kronfeld-Schor, 2011). However, when the golden spiny mouse is removed from the competitive environment in the field and placed in a semi-natural enclosure it displays nocturnal activity patterns (Gutman & Dayan, 2005), something that also occurs within the laboratory (Cohen, Smale, & Kronfeld-Schor, 2009). This suggests that the golden spiny mouse may represent a transitional state in which it maintains some features of an originally nocturnal ancestor but has undergone some changes in masking, such that it can be described as neither nocturnal nor diurnal.

In the laboratory, some other diurnal rodents can experience inversions in their behavioral patterns with the introduction of a running wheel. Plasticity in this artificial context has been observed in the *Octodon degus* (referred to below as a degus; Kas &

Edgar, 1999; Otalora, Vivanco, Madariaga, Madrid, & Rol, 2010), Mongolian gerbil (Weinert, Weinandy, & Gattermann, 2007), and the Nile grass rat (referred to below simply as a grass rat; Blanchong, McElhinny, Mahoney, & Smale, 1999; Blanchong & Smale, 2000). It should be noted that not all degus and grass rats are capable of this nocturnal activity pattern, and that the literature on daily rhythms and niche classification of Mongolian gerbils is conflicting (Weinert et al., 2007). However, when members of these species are night-active in the lab, they all have patterns of masking responses to light and darkness typically seen in nocturnal species (degus: Vivanco, Rol, & Madrid, 2010; grass rats: Redlin & Mrosovsky, 2004; Mongolian gerbils: Weinert et al., 2007). Although this work demonstrates the remarkable plasticity found in diurnal rodents, the context is highly artificial and it is difficult to know if it can provide insight into more fundamental mechanisms.

Neural Mechanisms of Masking

The first attempts to understand the neural mechanisms of masking in mammals focused on the identification of the input systems through which light might drive the behavioral response. Mrosovsky et al. (1999) initially noticed the impact of retinal degeneration on the masking response when they saw that rd/rd mice, which have a mutation that induced the loss of rods and cones as the animal aged, no longer showed positive masking in response to darkness but maintained the ability to respond to light. This indicated positive and negative masking responses are mediated by two different types of photoreceptive cells at the level of the eye. Further evidence for this segregation came from examination of the spectral sensitivities of the two masking

responses. Specifically, the negative masking seen in nocturnal mice is more sensitive to short-wavelengths while positive masking in these animals is induced by dimming medium-wavelength light in the range associated with the activation of rods and cones (Thompson, Foster, Stone, Sheffield, & Mrosovsky, 2008). Little was known, however, about the type of photosensitive retinal cell that mediates the negative masking seen in these animals, until the mid-2000s.

The search for the photoreceptors responsible for negative masking in nocturnal species involved many investigators because these cells were also believed to mediate photic influences on the circadian pacemaker and to induce the papillary light reflex (Gooley, Lu, Fischer, & Saper, 2003). The short-wavelength photoreceptive cells, referred to now as intrinsically photo-responsive retinal ganglion cells (ipRGCs), contain a newly discovered photopigment, melanopsin, and these cells were found to be the ones that project to the SCN (Gooley, Lu, Chou, Scammell, & Saper, 2001). In mice that have been genetically engineered to no longer express (knocked-out) the gene encoding of the protein melanopsin ($Opn4^{-/-}$), photic influences on circadian rhythms (Panda et al., 2002), the pupilary reflex (Lucas et al., 2003), and masking (Mrosovsky & Hattar, 2003) were all diminished. However, when these ipRGCs are completely eliminated in mice by knocking-in a toxin that kills the cells, mice no longer exhibit evidence of either positive nor negative masking (Guler et al., 2008). This work established that melanopsin-containing ipRGCs are the conduit that relays photic information into the brain to produce both positive and negative masking. Since the discovery of these cells, all examinations into the neural mechanisms of masking have focused on brain regions that receive input from them.

The melanopsin-containing ipRGCs project to many areas of the mouse brain (Hattar et al., 2006), including the suprachiasmatic nucleus (SCN), intergeniculate leaflet (IGL), dorsal lateral geniculate (DLG), and olivary pretectal nucleus (OPN). All of these areas have been investigated in nocturnal animals, to determine if they play a role in the mediation of masking responses to light. Such studies have led Mrosovsky and colleagues propose that multiple areas mediate masking of activity in response to light but that no single one of these areas is necessary for its maintenance (for review see Redlin, 2001). Based on their early work with rd/rd mice, Mrosovsky and colleagues targeted areas that were associated with vision. Specifically, these investigators have ablated the IGL (Redlin, Vrang, & Mrosovsky, 1999), DLG (Edelstein & Mrosovsky, 2001), and visual cortex (Redlin, Cooper, & Mrosovsky, 2003) and observed an actual increase in the masking response to light, with greater suppression of activity in lesioned animals. Recently, the effects of IGL lesions on masking have been examined in diurnal grass rats (Gall, Smale, Yan, & Nunez, 2013). Large lesions that encompassed both the IGL and part, or all, of the DLG produce a startling inversion of masking behavior, such that animals no longer positively masked to light but instead had a negative masking response. Therefore, here too there is no overall destruction of the masking behavior but an intriguing reversal in its direction, suggesting that the IGL-region is an important structure for maintenance of diurnal activity in grass rats.

Redlin and Mrosovsky (1999a) also destroyed the SCN of hamsters and saw no effect on masking in the context of an ultradian cycle, but they did not rule out the possibility that the SCN contributes to masking behavior. However, another study in which the same protocol was used, found that lesions directed at the SCN did block

masking in hamsters (Li, Gilbert, & Davis, 2005). One explanation of the discrepant findings proposed by Li et al. (2005) is that damage to areas of the hypothalamus that are innervated by retinal fibers that pass through the SCN (e.g. ventral subparaventicular zone, vSPZ) might be responsible for the effects they observed. The question of whether and how the SCN might play a role in masking in nocturnal mammals has thus not been settled, and nothing at all is known about this issue in diurnal species.

Finally, in his review of the neural mechanisms of masking, Redlin (2001) also proposes that the OPN might play an important role in masking. This region of the optic tectum lies downstream of the SCN and has reciprocal connections with the IGL. While no study has directly examined its role in regulation of general activity, one study has investigated the effects of OPN lesions on the ability of albino rats to experience a strain-specific form of masking, dark-induced REM sleep (Miller, Miller, Obermeyer, Behan, & Benca, 1999). Removal of the OPN greatly reduced the ability of animals to experience the induction of REM sleep by darkness, however, total sleep was not affected and activity was not monitored in that study.

Neither the SCN nor the OPN have been investigated in relation to their potential roles in masking in a diurnal species. Additionally, other brain regions such as the vSPZ and the pineal gland should be considered in the regulation of masking but have yet to be directly examined for their role in masking in either diurnal or nocturnal animals.

Overview of the chapters

In this dissertation, I present experiments that were designed to examine (1) what neural mechanisms drive the light-induced masking behaviors of a diurnal species and (2) how they may differ from those of their nocturnal counterparts. To address this question, I have used the diurnal grass rat and the nocturnal mouse to contrast responses of different brain regions to light at a time-of-day at which light induces activity in the nocturnal species and increases it in a diurnal one. Differences in the patterns of neural activation can provide a first step in the identification of brain regions that might be responsible for the behavioral differences between nocturnal and diurnal rodents (Chapter 2). Direct examination into the functions of one brain region in the grass rat, the vSPZ, will then be explored by ablating it with chemotoxic lesions (Chapter 3). In addition, I have removed the pineal gland in order to test the hypothesis that it may contribute to masking, as well as to diurnality more generally (Chapter 4). The immediate response of activity to light exposure, masking, is important in promoting the most advantageous activity pattern for an animal and identifying brain regions involved in the process of light-induced masking behavior will provide insight into the changes in the nervous system that have occurred during evolutionary transitions from one temporal niche to another. Therefore, these experiments examine a new avenue in the understanding of the evolution of diurnality.

CHAPTER 2

Acute effects of light on the brain and behavior of *Arvicanthis niloticus* and *Mus Musculus*

ABSTRACT

Photic cues influence daily patterns of activity via two complementary mechanisms: (1) entraining the internal circadian clock and (2) directly increasing or decreasing activity levels, a phenomenon referred to as “masking”. The direction of this masking response is dependent on the temporal niche an organism occupies; nocturnal animals typically decrease activity when exposed to light, while diurnal animals generally increase activity. Little is known about the neural mechanisms underlying these differences. Here, we directly compared the masking effects of light on behavior, and the activation of several brain regions by that light, in diurnal *Arvicanthis niloticus* and nocturnal *Mus musculus*. Each species displayed the temporal niche appropriate behavioral response to light, with the diurnal grass rats increasing their activity and the nocturnal mice decreasing their activity when presented with a 1 hr pulse of light two hours after lights off (ZT14, where ZT0 is lights on). The patterns of cFOS activation varied between the two species. Grass rats showed an increase in activation in all areas that receive retinal input while mice showed an increase in one of these regions (the suprachiasmatic nucleus), no change in others (the ventral subparaventricular zone, intergeniculate leaflet and lateral habenula) and a decrease in two (the olivary pretectal nucleus and dorsal lateral geniculate). In addition, cFOS was increased by light in three arousal-related brain regions (the lateral hypothalamus, dorsal raphe, and locus coeruleus) and one sleep-promoting region (the ventrolateral preoptic area) in grass

rats but not mice. Taken together, these results suggest the possibility that the retino-recipient and sleep/arousal-related brain regions that exhibit different patterns of responsiveness to light in the two species may contribute to differences in their behavior responses to photic stimuli.

INTRODUCTION

Light is a powerful environmental cue that can have a major impact on the daily behaviors and physiology of an organism (Aschoff, 1999). Specifically, light can have acute effects on behavior and physiology (masking effects) and it can impact the endogenous pacemaker (circadian rhythms effect; Mrosovsky, 1999; Redfern, Minors, & Waterhouse, 1994). Disentangling the influences of these two processes can be difficult in natural conditions because in environments with rhythmic alteration of light and darkness masking and circadian rhythms complement each other to coordinate the daily patterning of behavior and physiology (Redlin, 2001). Early circadian biologists devised experimental protocols to disentangle the influences of these two systems in the laboratory. However, in doing so they created a certain negative stigma associated with masking (Minors & Waterhouse, 1989a). This is evident in the original definition of the term, "…certain (sometimes overlooked) experimental conditions can *obscure* the real Zeitgeber-mechanism. We may call them masking conditions" (Aschoff, 1960). Masking, however, can reflect adaptive mechanisms that contribute to regulation of the daily patterning of activity, rather than processes that simply obscure the influences of circadian mechanisms.

Diurnal and nocturnal species differ with respect to the direction of the acute response of their locomotor activity to photic stimuli. Light produces an increase in activity (positive masking) in diurnal animals, whereas in a nocturnal species it typically decreases activity (negative masking; Mrosovsky, 1999). Many experiments have documented the suppression of activity by light in nocturnal mice (Butler & Silver, 2011; Morin, Lituma, & Studholme, 2010; Pendergast & Yamazaki, 2011), rats (Scheer, Ter

Horst, Van der Vliet, Buij, 2001), and hamsters (Redlin & Mrosovsky, 1999b). Masking effects of light on activity have been less well-studied in diurnal rodents, though they have recently been described in Nile grass rats (Redlin & Mrosovsky, 2004; Shuboni et al., 2012), degus (Vivanco et al., 2010), and Mongolian gerbils (Weinert et al., 2007).

Several approaches have been taken to investigate the neural basis of masking. One approach has been to examine effects of lesions of retino-recipient regions on acute behavioral responsiveness to photic stimuli. The first area to be examined was the suprachiasmatic nucleus (SCN), site of the primary circadian oscillator in mammals. Redlin and Mrosovsky (1999a) saw no effect of SCN lesions on masking in hamsters but they did not rule out the SCN as a possible contributor to masking. Another study found that lesions directed at the SCN did actually block masking (Li et al., 2005); these investigators propose that either the SCN or other hypothalamic areas that are innervated by retinal fibers that pass through it (e.g. ventral subparaventricular zone, vSPZ) play a major role in masking (Li et al., 2005). Effects of lesions of the intergeniculate leaflet (IGL) on masking have also been examined in two studies. Redlin et al. (1999) reported that IGL lesions actually increased negative masking in hamsters, while Gall et al. (2013) found that the lesions caused a change in the direction of the masking response of diurnal grass rats. Control grass rats showed positive masking of activity in response to a light pulse, while lesioned animals showed a negative masking response to light. In nocturnal rodents, lesions of the dorsal lateral geniculate nucleus (DLG; Edelstein & Mrosovsky, 2001) and visual cortex (Redlin et al., 2003) have also led to an increase in light-induced suppression of activity. Lesion studies have also implicated the retino-recipient olivary pretectal nucleus (OPN) in mediation of direct

effects of light on masking in nocturnal laboratory rats; specifically, light-induced REM sleep was attenuated by ablation of the OPN in these animals (Miller et al., 1999). Consideration of the effects of lesions of different retino-recipient areas of the brain led Mrosovsky and colleagues to propose that multiple areas mediate negative masking of activity by light in nocturnal species (Redlin, 2001).

A second approach to exploration of neural substrates of masking has focused on the recently discovered melanopsin-containing intrinsically photo-responsive retinal ganglion cells (ipRGCs) and the brain regions to which they project. When these cells are completely eliminated in mice, the animals no longer exhibit evidence of positive and negative masking (Guler et al., 2008). ipRGCs project to many areas of the brain (Hattar et al., 2006), one or more of which is likely to be functionally linked to masking.

Finally, several studies of nocturnal rodents have used the immediate early gene cFOS to characterize responsiveness to light of cells in regions that receive input from the retina, especially regions to which the ipRGCs project. Results from these studies have revealed region, species, and strain differences, summarized in Table 2.1. There are very few studies that have examined light-induced cFOS activation in diurnal species and most of these have focused exclusively on the SCN (Abe, Honma, Shinohara, & Honma, 1995; Krajnak, Dickenson, & Lee, 1997; M. M. Mahoney, Smale, & Lee, 2009; Schumann, Cooper, Hofmeyr, & Bennett, 2006). Only two study have examined light-induced cFOS beyond the SCN in a diurnal species, and this revealed an increase in cFOS in the peri-SCN region of Nile grass rats (Mahoney, Bult, & Smale, 2001) and the IGL of degus (Krajnak et al., 1997). Nothing is known about patterns of

responsiveness to photic stimuli in other brain region that receive input from ipRGCs in a diurnal species.

Here we present data collected with the aim of better understanding pathways that might contribute to masking, and where along these pathways differences between nocturnal and diurnal species might emerge. We first examined and directly compared the distribution of retinal projections in the grass rat and mouse to determine the extent to which regions known to receive input from melanopsin cells in the mouse also receive input from the retina in grass rats. We then examined the responsiveness of several of these regions to light administered at a time of day at which it suppresses activity in mice and increases it in grass rats. We also characterized the effects of this light on cFOS in sleep/arousal-related areas to determine how they may differ between the two species.

METHODS

Animals

Adult female grass rats were obtained from the breeding colony at Michigan State University and adult male CD1 mice were obtained from Charles River Laboratory (Wilmington, MA, USA). These animals were maintained on a 12:12 light-dark (LD) cycle with 300 lux of white light during the light phase and <1lux of red light during the dark phase. For the duration of the experimental procedures all animals were singly housed in Plexiglas cages (34x28x17 cm) equipped with an enrichment device (PVC, length: 8 cm, diameter: 6 cm); food (PMI Nutrition Prolab RMH 2000, Brentwood, MO) and water were available *ad libitum*. The Institutional Animal Care and Use Committee (IACUC) of Michigan State University approved these experimental procedures.

Table 2.1. Literature review of cFOS activation in response to light pulses in mice, rats, and hamsters. * Indicates that the study did not statistically analyze their data.

MICE

Author	Strain	Time	Duration	Areas	Trend
Brooks, 2011	C57BL/6J	CT16	30min	SCN SPZ	↑ ↑
LeGate, 2012	B6/129 F1 hybrid	ZT14	10min	SCN SPZ LHb	↑ ↑ ↑
Lima, 2003	C57BL/6J & C3H/HeJ	N/A	1hr	OPN	↑
Lupi, 1999	C57BL/6J	CT16 LP & CT17.5 Perfused	15min	SCN IGL	↑ (=)
Lupi, 2008	Control: C3H Mutant: C57BL/6 & 129/SvJ hybrid	ZT16	1hr	SCN VLPO	↑ ↑
Lupi, 2012	C3H/He	ZT16	15min	SCN IGL	↑ ↑
Mendoza, 2010	C57BL/6J	CT12	30min	LH (ORX) Raphe (5-HT)	(=) (=)
Tsai, 2009	C57BL/6 & 129/SvJ hybrid	ZT15	1hr	SCN VLPO	(=) (=), ↑ GAL+ cell
Huerta, 1999	C57BL/6J	CT16 &22	1 hr	SCN	↑*
Masana, 1996	C3H/HeN	CT2,6,10,14,18,&22	15min	SCN	↑
Delogu, 2012	Not Reported	ZT19	60min	SCN IGL	↑* ↑*

Table 2.1. (cont'd)

RATS					
Author	**Strain**	**Time**	**Duration**	**Areas**	**Trend**
Rusak, 1990	Not Reported	Subjective Night	30 or 60min	SCN IGL	↑* ↑*
Peters, 1996	Sprague-Dawley	Subjective Day & Subjective Night	2hr (FOS) & 30min (fos)	IGL	↑
Aronin, 1990	Sprague-Dawley	ZT4	4hr	SCN IGL	↑ ↑
Park, 1993	Sprague-Dawley	CT16	1, 1.5, 2, 3hr	SCN IGL	30-90min (=), 2+ ↑
Janik, 1992	Sprague-Dawley	CT14	2hr	SCN IGL	↑* ↑*
Cha, 2011	Sprague-Dawley	N/A	1hr	DLG	↑*
Juhl, 2007	Wistar	ZT6, ZT14, ZT19	90min	IGL	↑ ZT6, (=) ZT14 or 19
Prichard, 2002	Fischer F344 rats	ZT6 & 18 Control; ZT7 & 19 LP	60min	OPN IGL DLG	(=) midday, ↑ midnight ↑ midday and midnight (=)

Table 2.1. (cont'd)

HAMSTERS					
Author	**Strain**	**Time**	**Duration**	**Areas**	**Trend**
Rusak, 1990	Not Reported	Subjective Day & Subjective Night	30 or 60min	SCN IGL	↑ night, (=) day ↑ night, (=) day
Janik, 1995	Syrian	CT18	30min	SCN IGL	↑ ↑
Marchant, 1999	Syrian	CT18.5	15 min	SCN IGL DR	↑ ↑ ↑
Muscat, 2006	Golden	CT19	5 min	SCN IGL	↑ ↑
Zhang, 1993	Golden	CT19	5 min	SCN IGL SPZ	↑* ↑* ↑*
Zhang, 1996	Golden	CT19	5 min	SCN	↑

* Indicates that the study did not quanitatively analyze the data.

Experimental Procedures

Experiment 1: Mapping of retinal projections.

We used Cholera Toxin Beta-subunit (CTβ) injections into the eye to map all retinal projections in a grass rat (n=1) and mouse (n=1). Low salt CTβ (List Biological, Campbell, CA, USA) was diluted to a concentration of 3 mg/mL in a mixture of 1.8% DMSO and saline. The grass rat or mouse was deeply anesthetized using isoflorane (2.5-5%, Abbott Laboratories, IL, USA), the eye was then protruded from the socket and 5 µL of CTβ was injected into the posterior chamber using a 10 µL Hamilton syringe (Hamilton, Reno, Nevada, USA). The injection was given over a thirty-second period and the needle was left in the eye for an additional minute to ensure the diffusion of the substance into the vitreous humor. To obtain maximal labeling, both eyes were injected with the tracer. One week after the eye injections, the animals were deeply anesthetized using an i.p. injection of sodium pentobarbital (400 mg/kg). The animals were then transcardially perfused with 0.1M phosphate buffered saline (PBS, pH 7.2) and then a 4% paraformaldehyde solution in PBS, also containing 0.075M lysine and 0.01M sodium periodate (PLP, Sigma-Aldrich, St. Louis, MO, USA). The brains were post-fixed for an additional 4 hrs in the PLP solution; they were then transferred into sucrose for a minimum of 24 hrs, after which they were cut along the coronal plane at 30 µm using a freezing-microtome. These sections were divided into three series and stored in cyroprotectant at -20℃ until further processing.

Experiment 2: Behavioral and cFOS responses to nighttime light

Eight grass rats and ten mice were used in this experiment. Activity levels were monitored via infrared motion detectors (IRs, Visonic Tel Aviv, Israel) that were attached to each cage lid near the food and water. These monitors function by using infrared beams that, when broken, trigger a count; all of the counts were recorded with the VitalView Program (Minimitter, Bend, OR, USA) on a personal computer in an adjacent room. After two weeks of baseline data were collected, the grass rats and mice were both randomly assigned to either a control group (grass rat: n=4, mice: n=5) or a group that was exposed to a light pulse (LP; grass rats: n=4, mice: n=5). All of these animals were perfused (see above) at zeitgeber time (ZT) 15. Animals in the LP group were exposed to a pulse of light for an hour prior to sacrifice (ZT14-15). All grass rats in this experiment were also injected bilaterally with cholera toxin the week before sacrifice, but their brains were not processed for visualization of the tracer.

Immunocytochemistry (ICC)

Experiment 1: Mapping of retinal projections

One series of sections was reacted for the CTβ tracer. Unless otherwise mentioned, free-floating sections were incubated at room temperature and washed three times between incubations (10 minutes/wash) with 0.01M PBS (pH 7.2). The initial incubation was preceded by six 10-minute rinses in PBS to remove excess cryoprotectant. Sections were then blocked with 5% normal horse serum (Vector Laboratory, Burlingame, CA, USA) in PBS and 0.3% Triton X-100. Tissue was then incubated with goat anti- CTβ (1:100,000, List Biological) at 4℃ on a shaker f or 24 hrs. The tissue was then incubated with a biotinylated horse anti-goat secondary (1:100,

Vector Laboratory, Burlingame, CA, USA) and then avidin-biotin peroxidase complex (ABC) with the ABC Vectastain Kit (Vector Laboratory, Burlingame, CA, USA). The final reaction of the tissue used 3,3'-diaminobenzidine (DAB, 0.5 mg/mL, Sigma-Aldrich) and 30% hydrogen peroxide in a Trizma buffer (Sigma-Aldrich) to visualized the retinal fibers as a dark brown stain. After two minutes, the reaction was stopped with three rinses of 0.01M PBS, 10min/rinse. The sections were then mounted onto gelatin-coated slides, dehydrated, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA).

Experiment 2: *Behavioral and cFOS responses to nighttime light*

One series of sections from each animal (i.e. every 3rd section) was processed with immunohistochemistry to visualize the distribution of the protein cFOS. The protocol for grass rats followed the same steps outlined for the CTβ reaction. In brief, sections were incubated in (i) 5% normal donkey serum (Jackson ImmunoResearch, West Grow, PA, USA), (ii) the primary rabbit anti-cFOS antibody (1:50,000, Santa Cruz Biochemistry, Santa Cruz, CA, USA), (iii) biotinylated donkey anti-rabbit antibody (1:200, Jackson ImmunoResearch) and (iv) the ABC complex (Vector Laboratory). From this point the procedure differs from that used for the CTβ. Sections were rinsed in a 0.14M acetate buffer (pH 7.2) three times, 10 min/wash. To visualize the protein, a mixture of DAB (0.5mg/mL) and nickel sulfate in 0.14M acetate buffer (pH 7.2) was reacted with 3% hydrogen peroxide to produce a bluish-purple stain. Once the reaction was complete (6 minutes), the tissue was washed three times in PBS, 10 min/wash. Sections were mounted, dehydrated, and coverslipped with dibutyl phthalate xylene (Sigma-Aldrich).

The procedure for processing tissue from the mice followed similar steps except that: (1) the tissue was rinsed in 0.1% PBT (PBS with .01% Triton X-100) rather than PBS, (2) the concentration of the primary antibody, rabbit anti-cFOS, was higher (1:20,000), and (3) the tissue was incubated over-night in the ABC solution. Finally, the DAB reaction was carried out in a Trizma buffer (as in the CTβ protocol described above) and lasted for only 3 minutes.

A second series of brain sections from each animal was stained with cresyl violet and used to delineate regions of interest for analysis.

Data Analysis

Experiment 1: Mapping of retinal projections

Detailed maps of all sections containing retinal fibers were drawn and corresponding photographs were taken. The drawings were constructed using a light microscope (Leitz, Wetzlar, Germany) equipped with a drawing tube; drawings were made using a low power objective but fibers were confirmed using a higher power objective. From the complete map, areas of interest were identified and photographed using a CCD camera (CX900, MBF Biosciences, Williston, VT, USA) attached to a light microscope (Ziess, Gottingen, Germany).

Experiment 2: Behavioral and cFOS responses to nighttime light

To analyze the behavioral data, Vital View files were converted into actograms via ClockLab (Actimetrics, Wilmette, IL, USA) and raw data were transferred into Microsoft Excel. The actograms provided visual confirmation of masking behavior during

the light pulse, while Excel allowed for quantitative assessment of the data. To statistically compare activity between groups, the raw data were corrected for differences in motion detector sensitivity by (1) converting the data into percentages (activity during the hour before sacrifice/24 hr of baseline activity) and (2) subjecting them to an arcsine transformation. Effects of light on behavior were examined in each species by comparing these values from light pulsed and control animals with independent-sample t-tests using SPSS.

For the analysis of cFOS we focused primarily on areas that receive retinal input and/or are associated with sleep/arousal. Observers that were blind to which experimental group the animal had been in did counts of numbers of cFOS+ cells in all brain regions. A light microscope equipped with a drawing tube was used to produce bilateral maps of labeled cells in all areas, with the exception of the dorsal raphe (DR), which had one centrally located counting box, from at least one section. Counts were done on two sections for the SCN, vSPZ, IGL, DLG, the ventrolateral preoptic nucleus (VLPO), the lateral hypothalamus (LH), locus coeruleus (LC) and DR. Counting boxes described in earlier reports were used to delineate regions sampled in the VLPO (Novak, Smale, & Nunez, 1999), vSPZ (Schwartz, Nunez, & Smale, 2004), LH (Martinez, Smale, & Nunez, 2002; Nixon & Smale, 2004), LC (Castillo-Ruiz & Nunez, 2011), and DR (Castillo-Ruiz & Nunez, 2011). For the remaining regions whole areas were identified and counted. The boundaries of the SCN and OPN were easily visualized, while the VMH, IGL and DLG were delineated with the aid of the nissl stained tissue. Independent sample t-tests were used to determine if numbers of FOS+

cells differed between light-exposed and control groups; counts from each region, and from each species, were analyzed separately.

RESULTS

Experiment 1: Mapping of retinal projections

Drawings of retinal fibers in a sequence of sections, and photomicrographs of such sections, are shown for grass rats and mice in Figure 2.1. Here, we focus on retinal projections outside the primary optic tract. The first fibers observed were in the VLPO; both species had fibers in this region but they were sparse (3 to 6 per section) (Figure 1A-B, section 1). Grass rats, unlike mice, also had staining medial to the VLPO, in the ventromedial preoptic nucleus. In subsequent sections (Figure 2.1A-B, sections 2-4) dense retinal fibers could be seen across the full rostral-caudal extent of the SCN. These findings are similar to those previously reported for the grass rat (Smale & Boverhof, 1999) and mouse (Hattar et al., 2006; Mikkelsen, 1992). In the most rostral section containing the SCN, fibers also became visible within the supraoptic nucleus (SON, Figure 2.1A-B, section 2) in both species; innervation of the SON increased as sections progressed caudally. Fibers along this pathway extended beyond the SON into the amygdaloid complex and the lateral hypothalamus (LH, Figure 2.1A-B, section 5). The mice appeared to have a few more fibers extending toward the amygdaloid complex but were otherwise identical to the grass rats. In the caudal sections of the SCN (Figure 2.1A-B, section 3-4), a group of fibers radiated dorsally from the nuclei, extending into the vSPZ; this was the case in both species, however, in grass rats these fibers extended further beyond the SCN than they did in mice. Many fibers were also

Figure 2.1. Line drawings of retinal projections in *Mus musculus* and *Arvicanthis niloticus.* Animals were injected bilaterally with the tract tracer CTβ and their brains were processed using immunocytochemistry. Sections 1-8 depict the rostral to caudal progression of projections in the grass rat (A) and mice (B). A magnification of sections 1-5 (C), illustrates that mice had fewer fibers within the vSPZ than grass rats, section 3. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

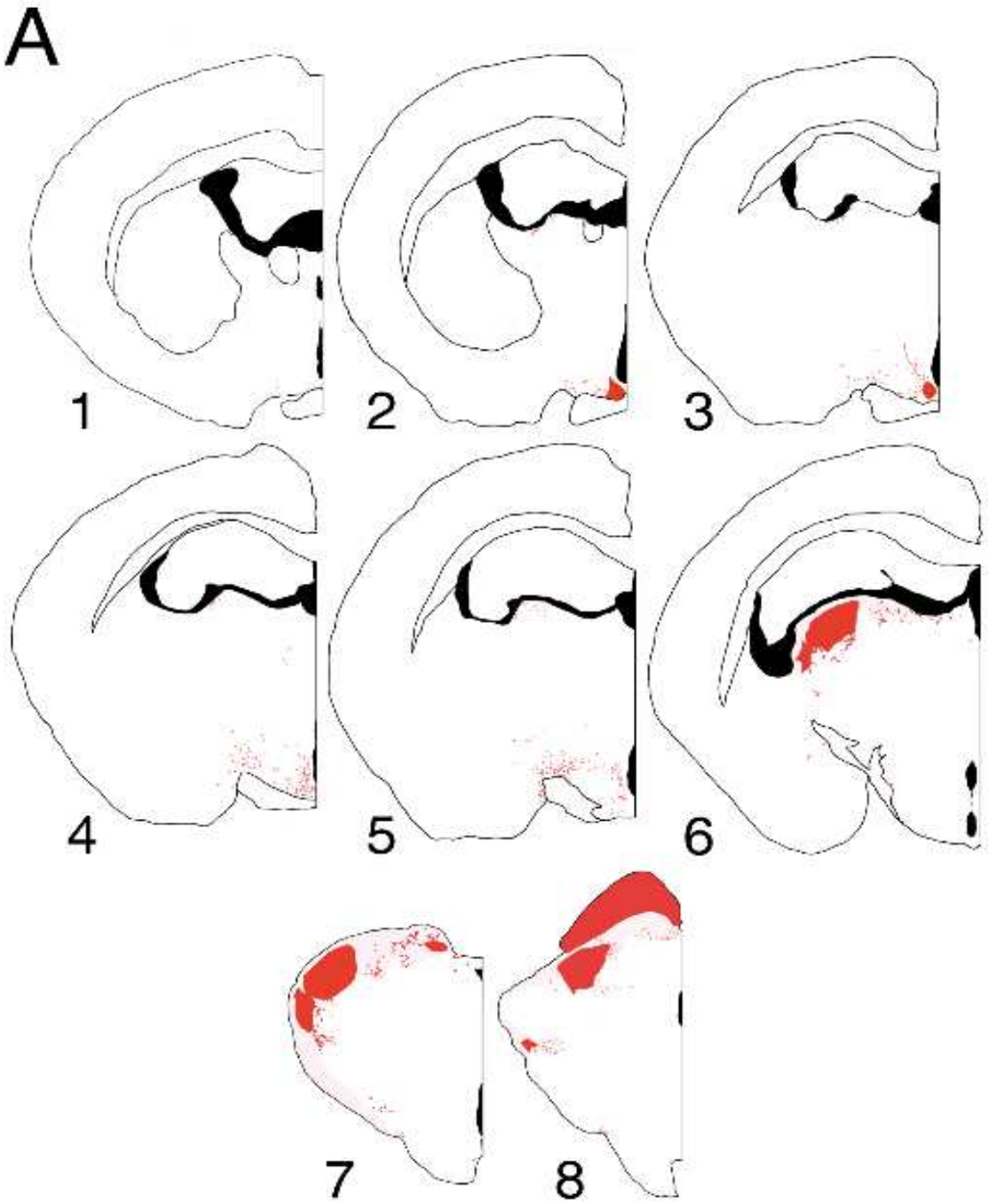
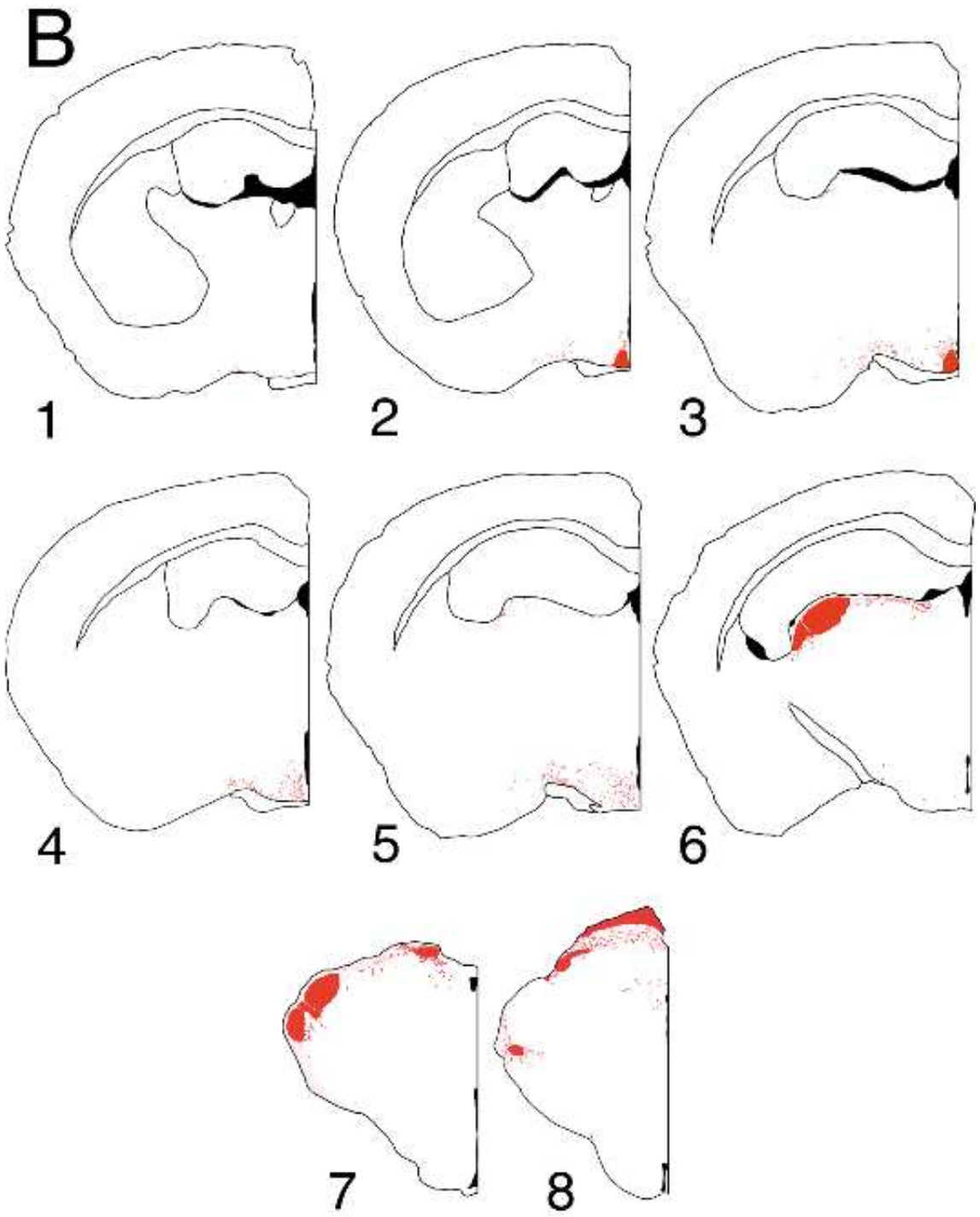


Figure 2.1. (cont'd)



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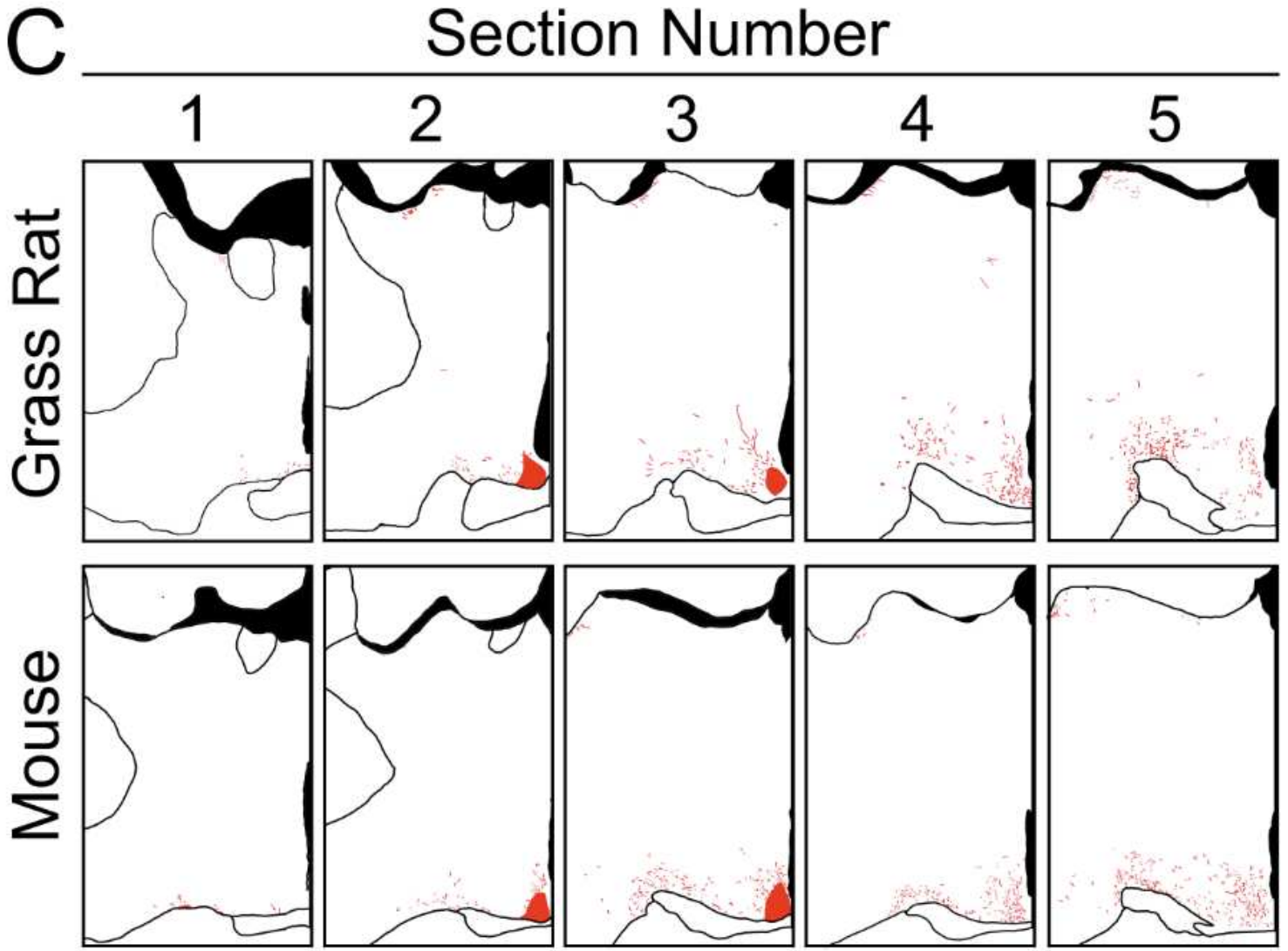
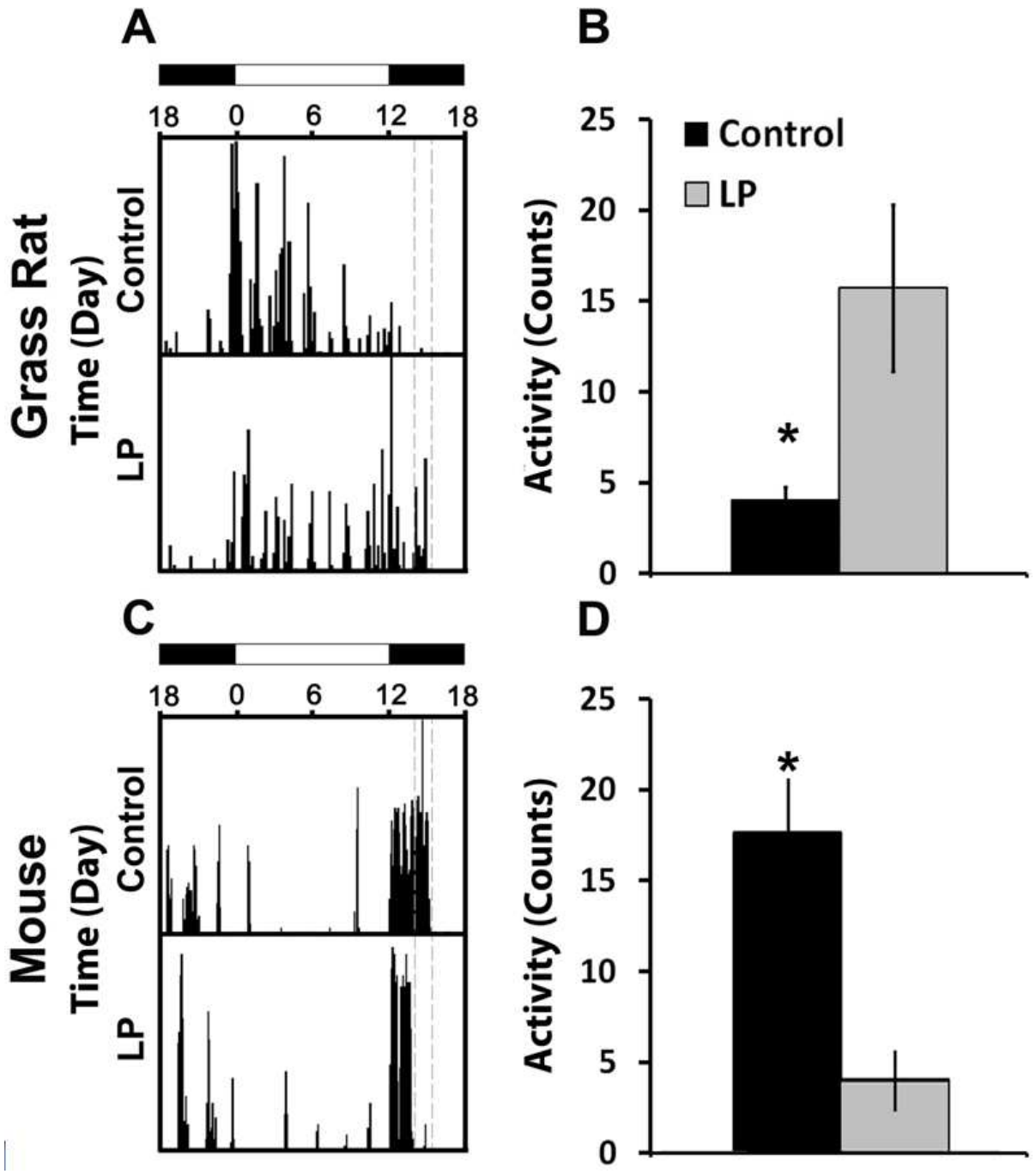


observed within the retrochiasmatic area (RCh, Figure 2.1A-B, section 5), and extending dorsally into the anterior hypothalamic area (AHA). As sections progressed caudally, hypothalamic innervations became restricted to the area immediately dorsal to the optic tract (opt). In grass rats, fibers were observed in the dorsomedial hypothalamic nucleus (DMH) but not in the ventromedial hypothalamic nucleus (VMH). The distribution of fibers in the DMH was similar in the two species, but in mice these fibers extended into the anterior sections of the VMH. Dense retinal fibers were seen in all regions of the geniculate complex of both species (Figure 2.1A-B, section 6-7); (i.e. the dorsal lateral geniculate nucleus (DLG), intergeniculate leaflet (IGL) and the ventral lateral geniculate (VLG). The DLG appeared first; at this level, fibers also began to extend toward the midline. Progressing caudally, we saw one fiber in the lateral habenula (LHb) of each species, and in the paraventricuar thalamus (PVT), 2-3 fibers were visible in the grass rat and 1-2 fibers in the mouse. Fibers were also observed medial to the DLG where they became denser in both species as the OPN appeared (Figure 2.1A-B, section 7). These nuclei migrated laterally as the superior colliculus emerged with very dense staining of the tracer (Figure 2.1A-B, section 8). Overall, the distribution of the retinal fibers was very similar in the grass rat and mouse; the only regions that differed substantially were the vSPZ, where there were more labeled fibers in the grass rat, and VMH, where there were more fibers in the mouse than grass rat.

Experiment 2: Behavioral and cFOS responses to nighttime light

Behavior

Figure 2.2. Behavioral masking response to 1 hr of light exposure. Grass increased activity as observed in a representative actogram (A) and group data (B). The opposite behavior was observed in mice as both a representative actogram (C) and group data (D) had a decrease in activity. An asterisk (*) indicates significance $p < .05$.

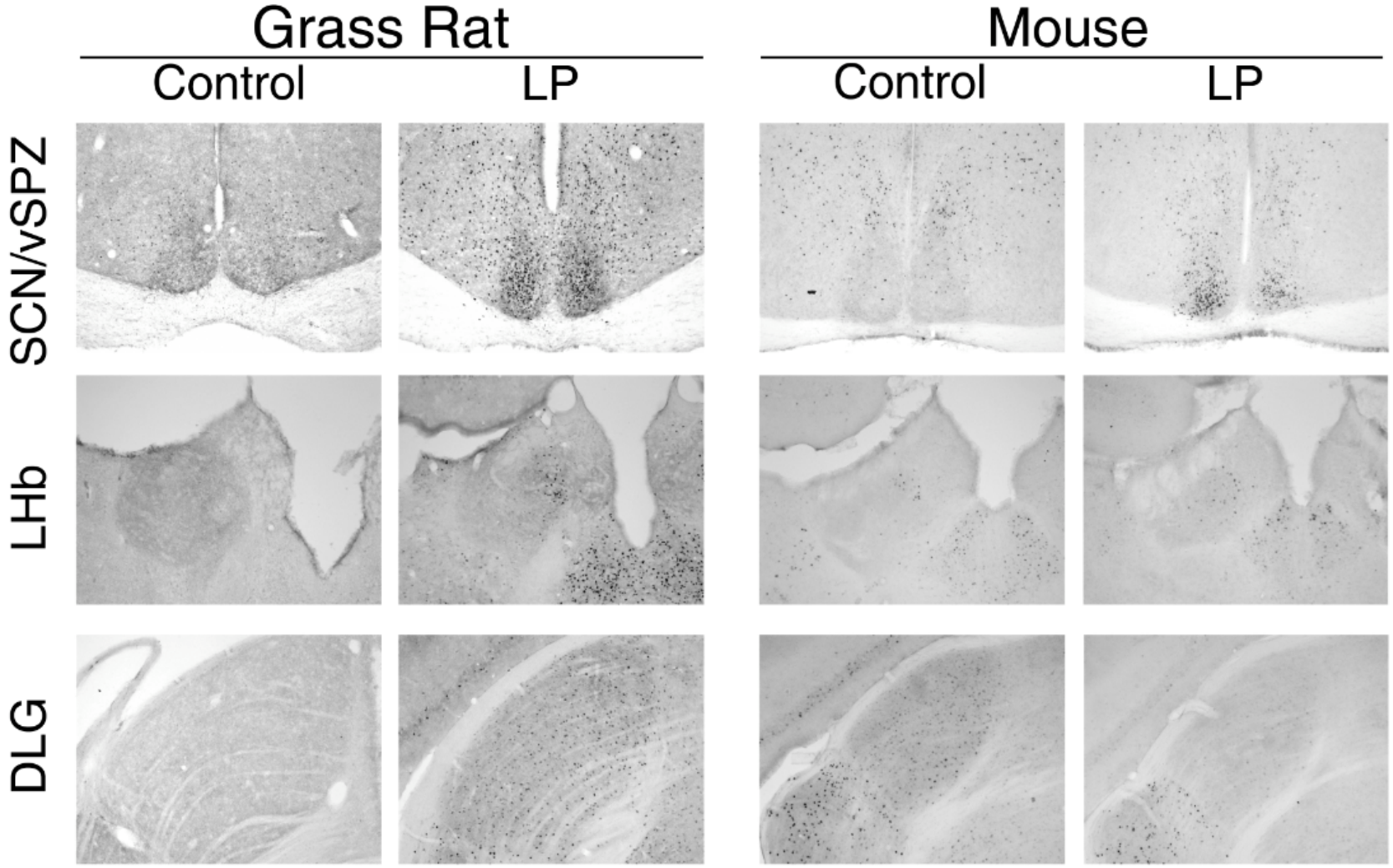


Exposure of grass rats to a 1 hr light pulse at ZT14 induced a marked increase in general activity (Figure 2.2A). Specifically, among control animals, 4.69±0.67% of daily activity occurred during the hour beginning at ZT14, whereas among animals exposed to 1 hr of light at ZT14 this figure was significantly higher, at 15.72±4.59% ($t(5)=2.74$, $p=0.041$). The opposite response was observed in the mice. In this case, the percentages of daily activity occurring during the hour beginning at ZT14 were 17.66±3.52% among control mice but only 4.06±1.53% among light-exposed animals ($t(8)=3.75$, $p=0.006$; Figure 2.2B).

cFOS expression in Retino-Recipent Regions

In our examination of cFOS responses to light in retinorecipient regions of mice and grass rats we found four basic patterns: (1) light induced an increase in cFOS in both species, (2) neither species responded to the light, (3) the responses of the two species were in opposite directions, and (4) a cFOS response was seen in grass rats but not mice. The first pattern was evident in the SCN, where the number of cFOS+ cells increased in response to the 1 hr light pulse in both species (Grass Rat: $t(5)=4.04$, $p=0.01$ and Mouse: $t(8)=4.77$, $p=0.001$; Figure 2.3). The second pattern occurred in the VMH, where neither species responded to the light; (Figure 2.3; Grass rat: ($t(5)=1.10$, $p=0.32$; Mouse: $t(8)=0.67$, $p=0.59$). The VMH was visually striking in grass rats exposed to light, as it was relatively void of cFOS+ cells compared to the highly reactive surrounding regions (the arcuate nucleus and DMH, Figure 2.3). The two species exhibited opposite patterns of response to light in the DLG and OPN (Figure 2.3). Specifically, light induced an increase in cFOS in both areas in grass rats

Figure 2.3. Photomicrographs of cFOS in retino-recipient regions. Grass rats on the right, have controls with relatively low cFOS activation while the light pulse (LP) have higher levels with the exception of the VMH that remained unchanged. The mice on the left, had an increase in cFOS activation after the LP (SCN), no change between control and LP (vSPZ, LHb, IGL, and VMH), or a decrease in cFOS activation after LP (DLG and OPN).



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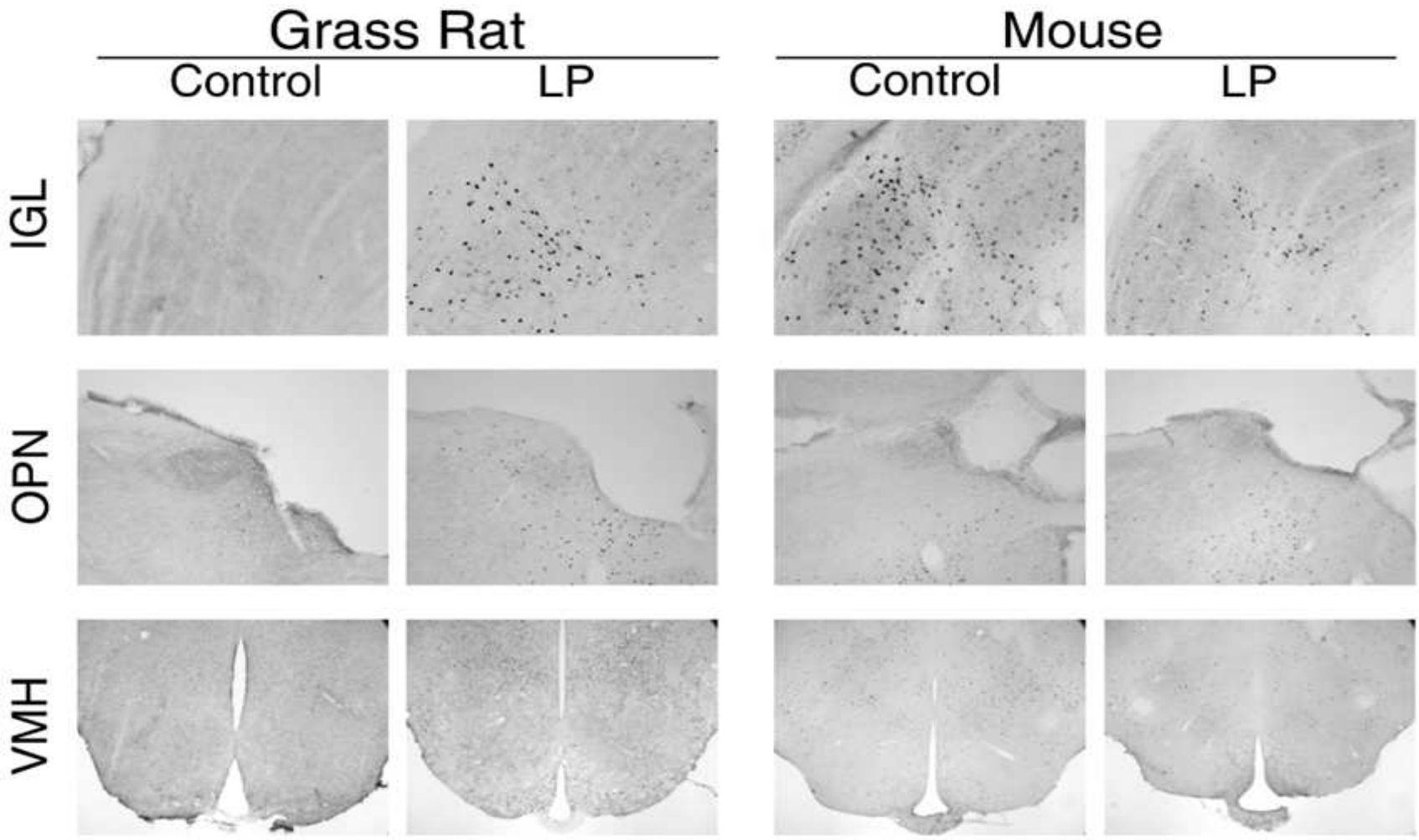
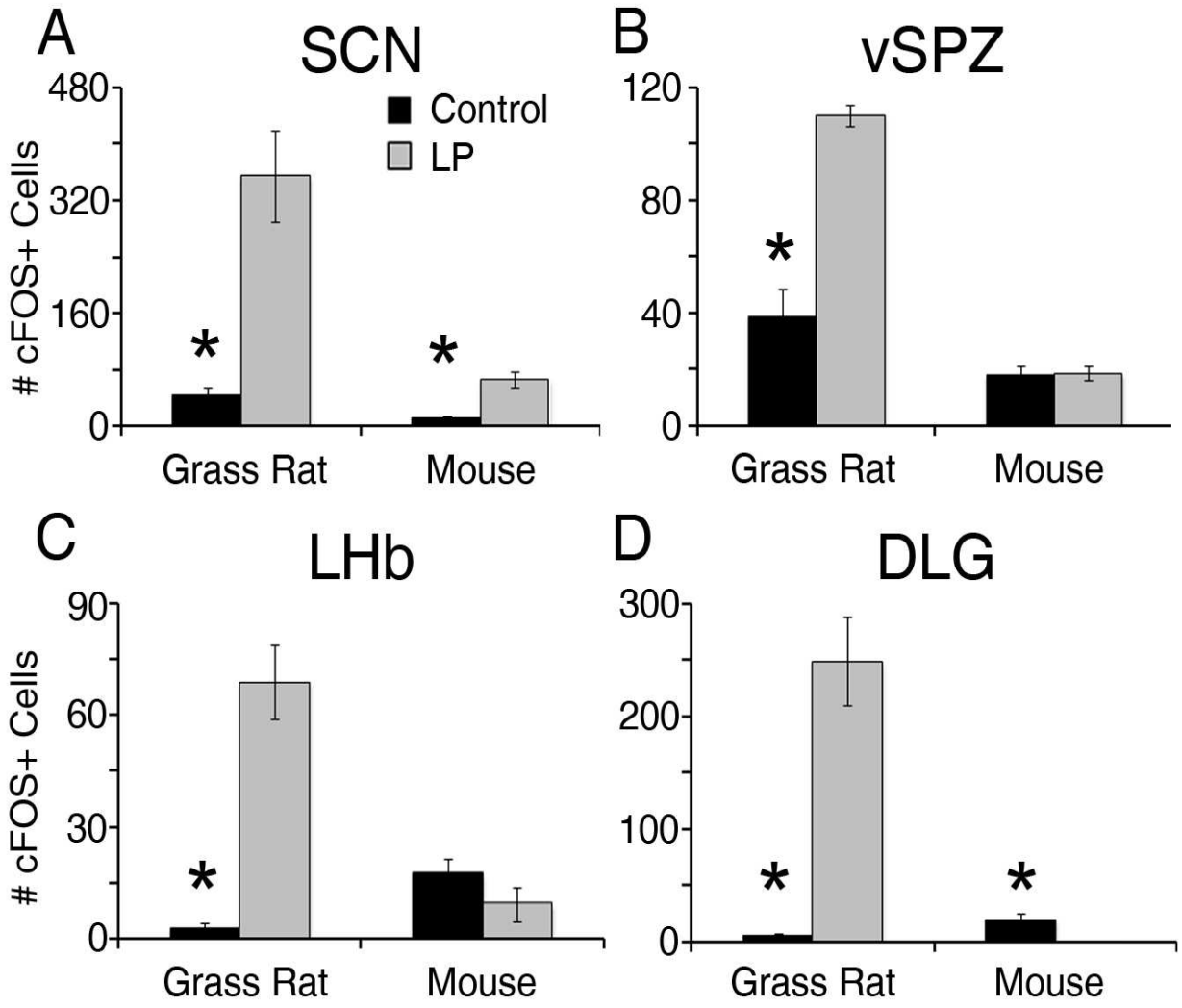


Figure 2.4. Patterns in cFOS expression in retino-recipient regions. Panel A shows a significant increase in activation within the SCN. Three regions, the vSPZ (B), LHb (C) and IGL (E) respond with a significant increase in grass rats but not in the mice. Two regions, the DLG (D) and OPN (F) have an opposite response to light exposure with mice experiencing a decrease and grass rats an increase in activation. Panel G shows no response within the VMH of either species. An asterisk (*) indicates significance $p < .05$.



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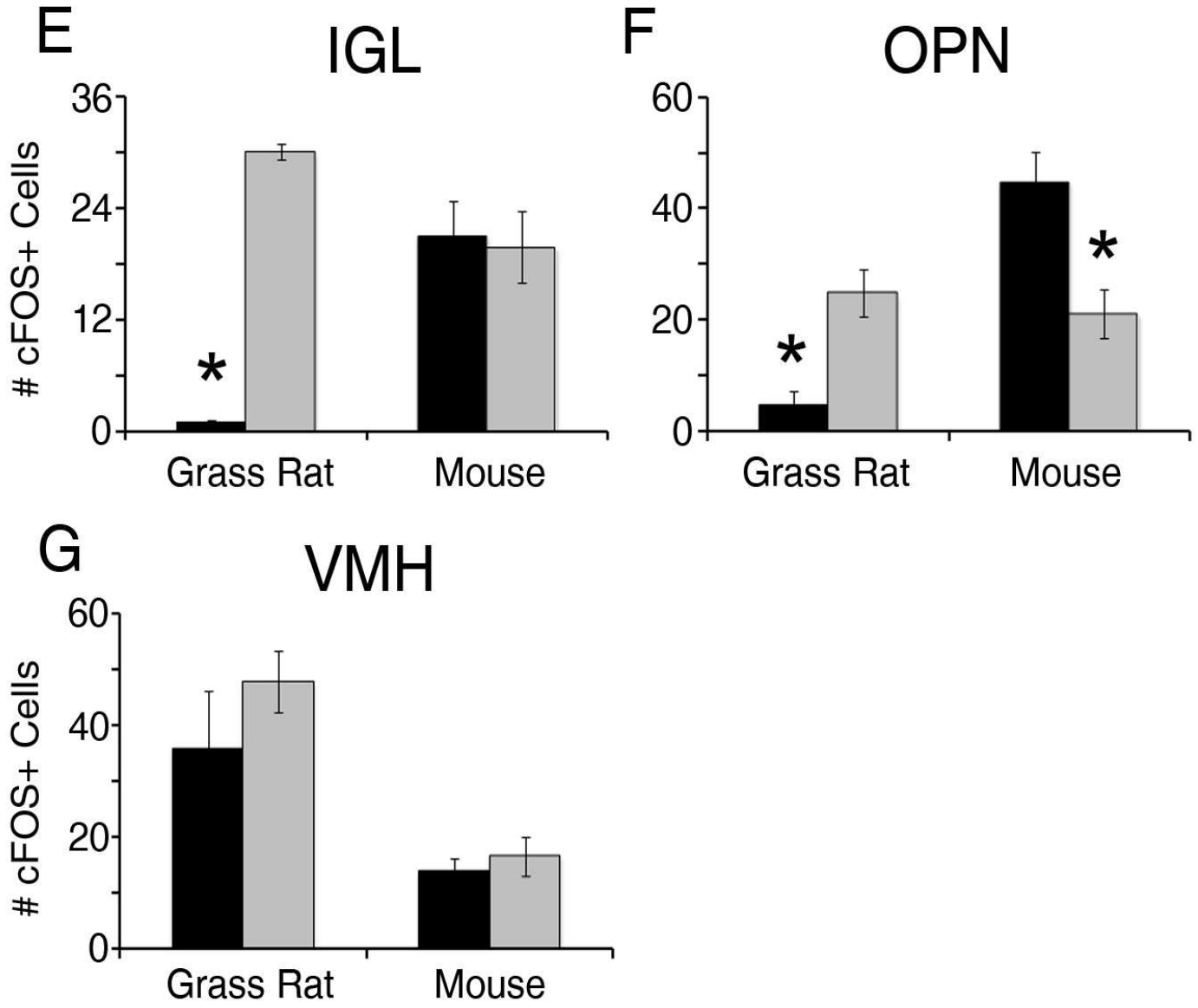


(DLG: $t(5)=3.61$, $p=0.012$; OPN: $t(5)=3.61$, $p=0.012$), and a decrease in these regions in mice (DGL: $t(8)=4.308$, $p=0.003$ and OPN: $t(8)=3.486$, $p=0.008$). Closer inspection revealed that several of the control mice had very high concentrations of FOS+ cells in these two regions; this was the case for the DLG in three of five mice (Figure 2.5) and the OPN in four of five mice (Figure 2.6).

In all other retinorecipient areas that we examined light induced an increase in cFOS in grass rats and had no significant effect in mice. The grass rats responded to light with an increase in activation within the vSPZ ($t(5)=7.57$, $p<0.01$; Figure 2.3), IGL ($t(5)=31.22$, $p<0.001$; Figure 2.3), and LHb ($t(5)=5.67$, $p<0.01$; Figure 2.3). In mice, light had no effect on cFOS in these regions (vSPZ: $t(8)=0.12$, $p=0.908$; IGL: $t(8)=0.22$, $p=0.828$; LHb: $t(8)=1.33$, $p=0.220$).

cFOS expression in Arousal/Sleep-Related Regions

As light-induced masking has been shown to inhibit sleep in diurnal species and induce it in nocturnal ones, we examined regions that are associated with sleep and arousal. In the grass rat, all arousal-inducing areas examined showed an increase in cFOS+ cells after the light pulse; this included the DR ($t(5)=7.67$, $p<0.01$; Figure 2.7) and the LC ($t(5)=7.11$, $p<0.01$; Figure 2.5). Among mice, there was no response to a light pulse in the DR ($t(8)=0.774$, $p=0.461$) or the LC ($t(8)=1.753$, $p=0.118$). In the LH (Figure 2.7), where orexin neurons are found, control grass rats had significantly lower numbers of cFOS+ cells than grass rats exposed to the light pulse ($t(5)=11.50$, $p<0.001$). Among mice cFOS did not differ in the two groups ($t(8)=0.66$, $p=0.527$).

Figure 2.5. Photomicrographs of the dorsal lateral geniculate nucleus for all mouse subjects. On the left, 3 control mice have very high levels of cFOS (MM06, MM09 & MM10) while the remaining two have moderate levels of expression (MM07 & MM08). On the right, all LP animals have low levels of cFOS expression.

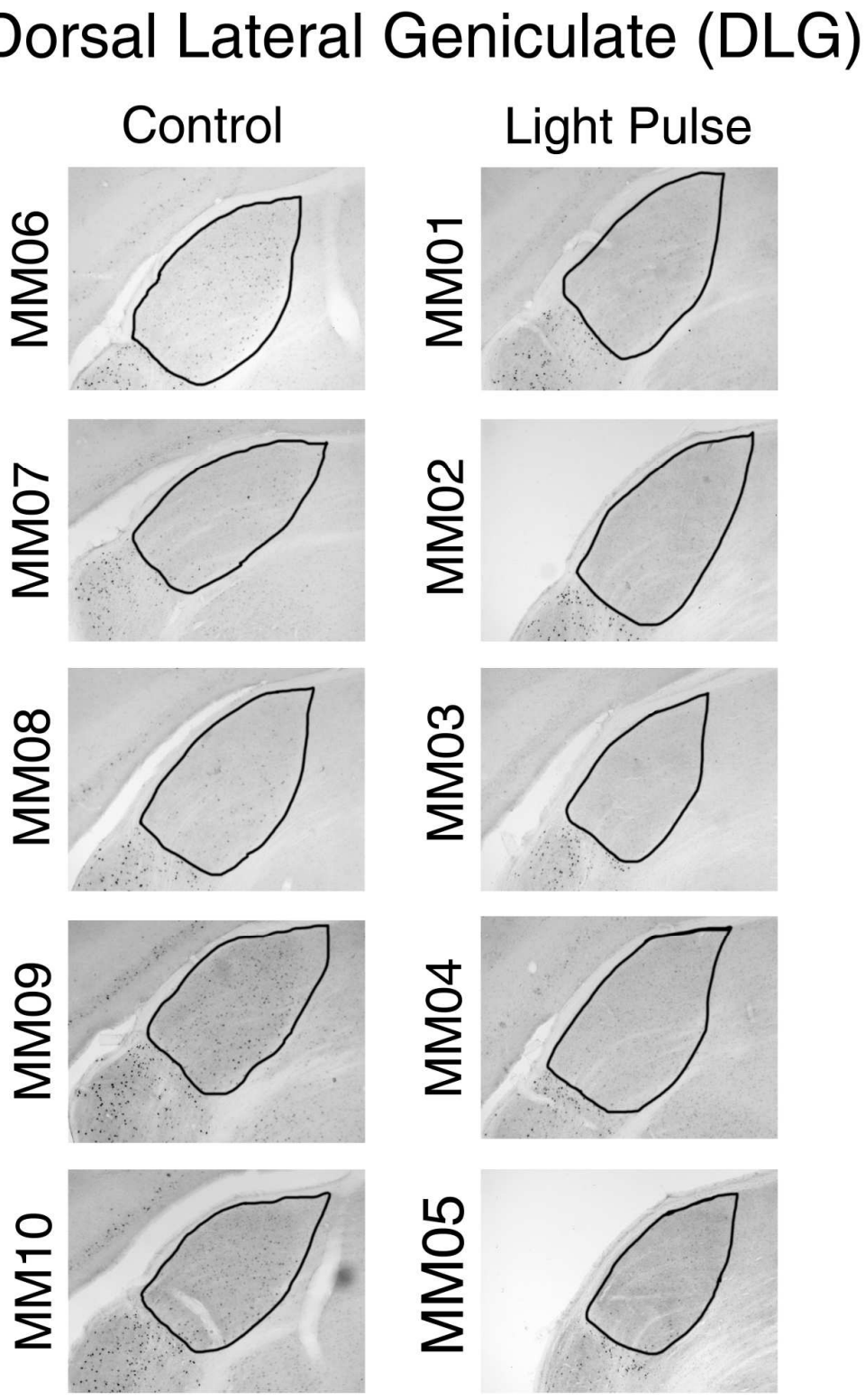


Figure 2.6. Photomicrographs of the olivary pretectal area for all mouse subjects. On the left, 4 control mice have high levels of cFOS (MM06, MM07, MM09 & MM10) while all but MM4 of the LP animals (Right) have low levels of cFOS expression.

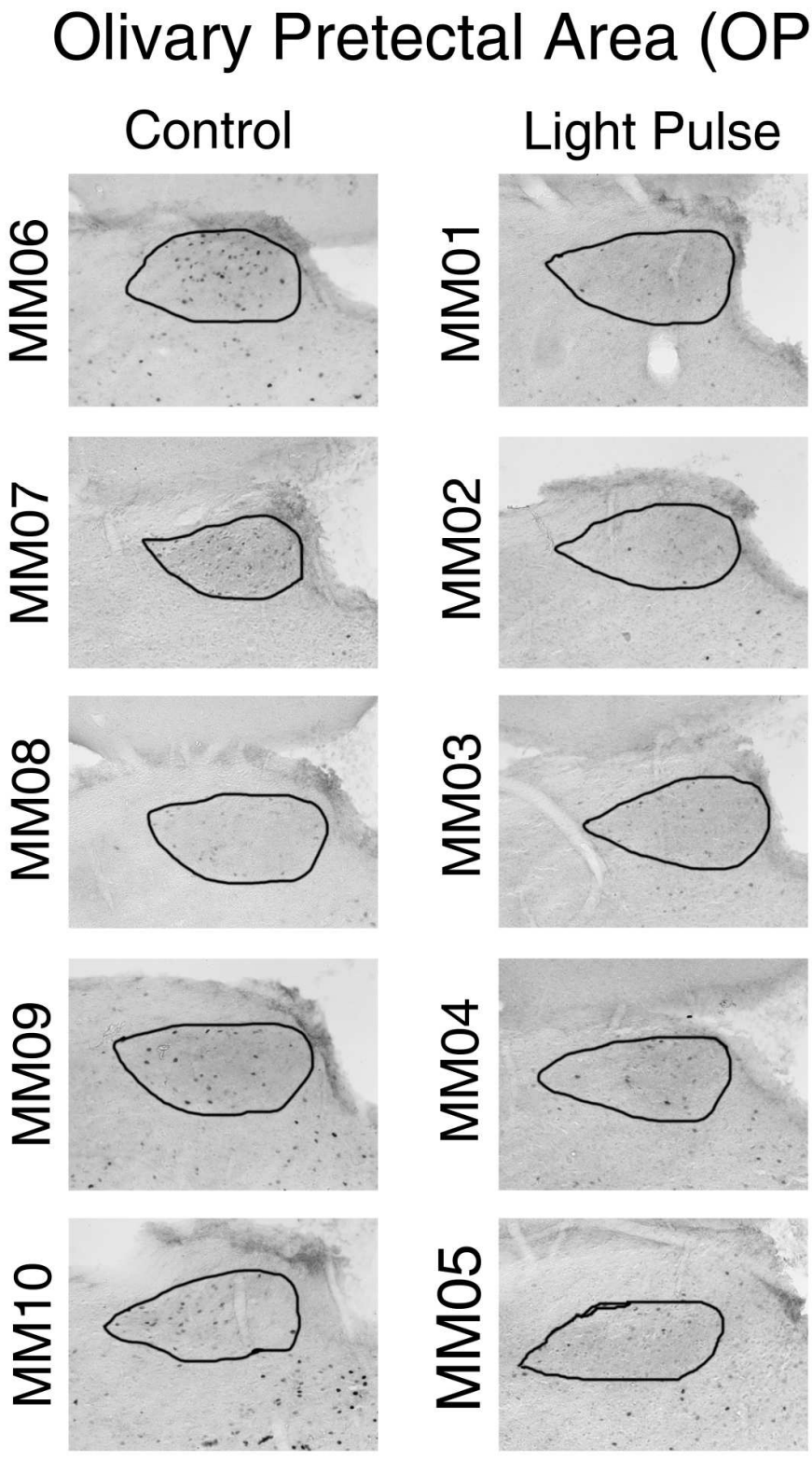
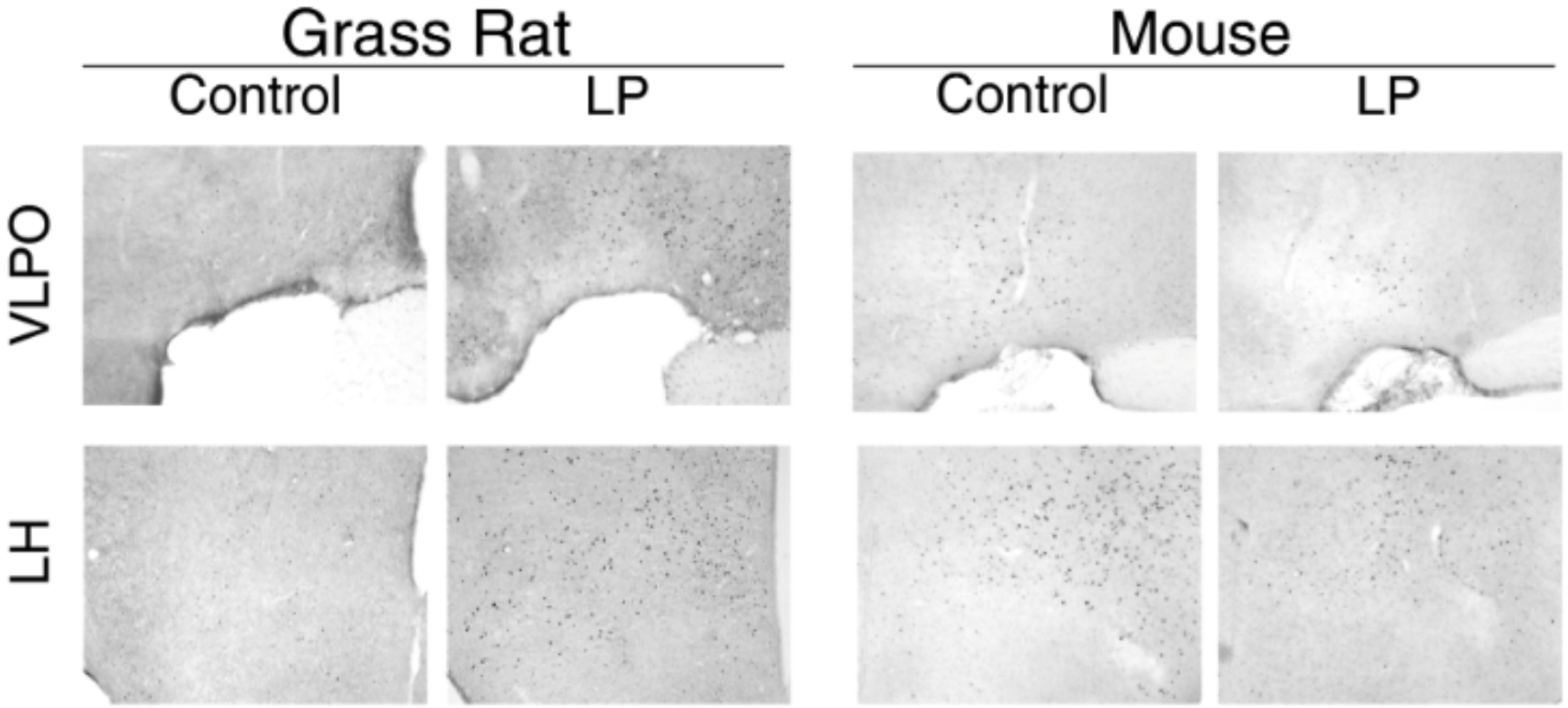


Figure 2.7. Photomicrographs of cFOS in arousal/sleep-related regions. Grass rats on the right, have controls with relatively low cFOS activation while the light pulse (LP) animals have higher levels in all areas examined (VLPO, LH, DR, LC). The mice on the left, had no change between control and LP for all these areas.



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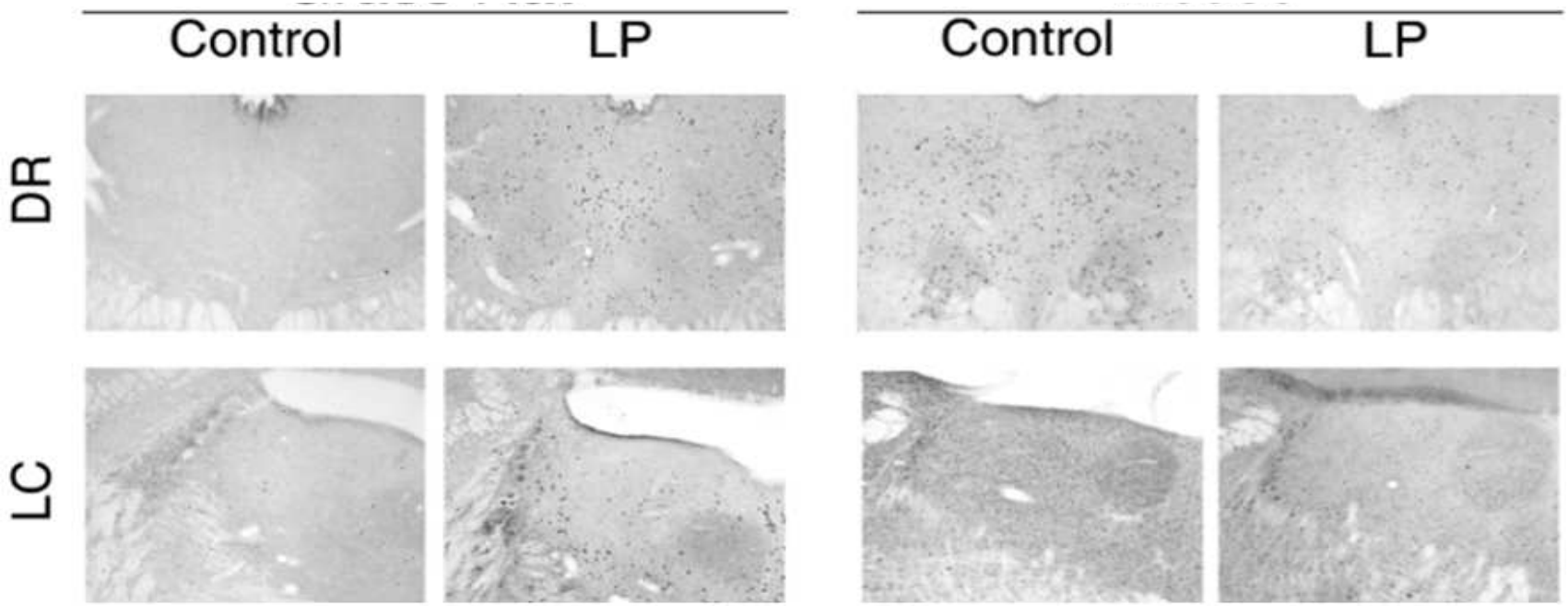
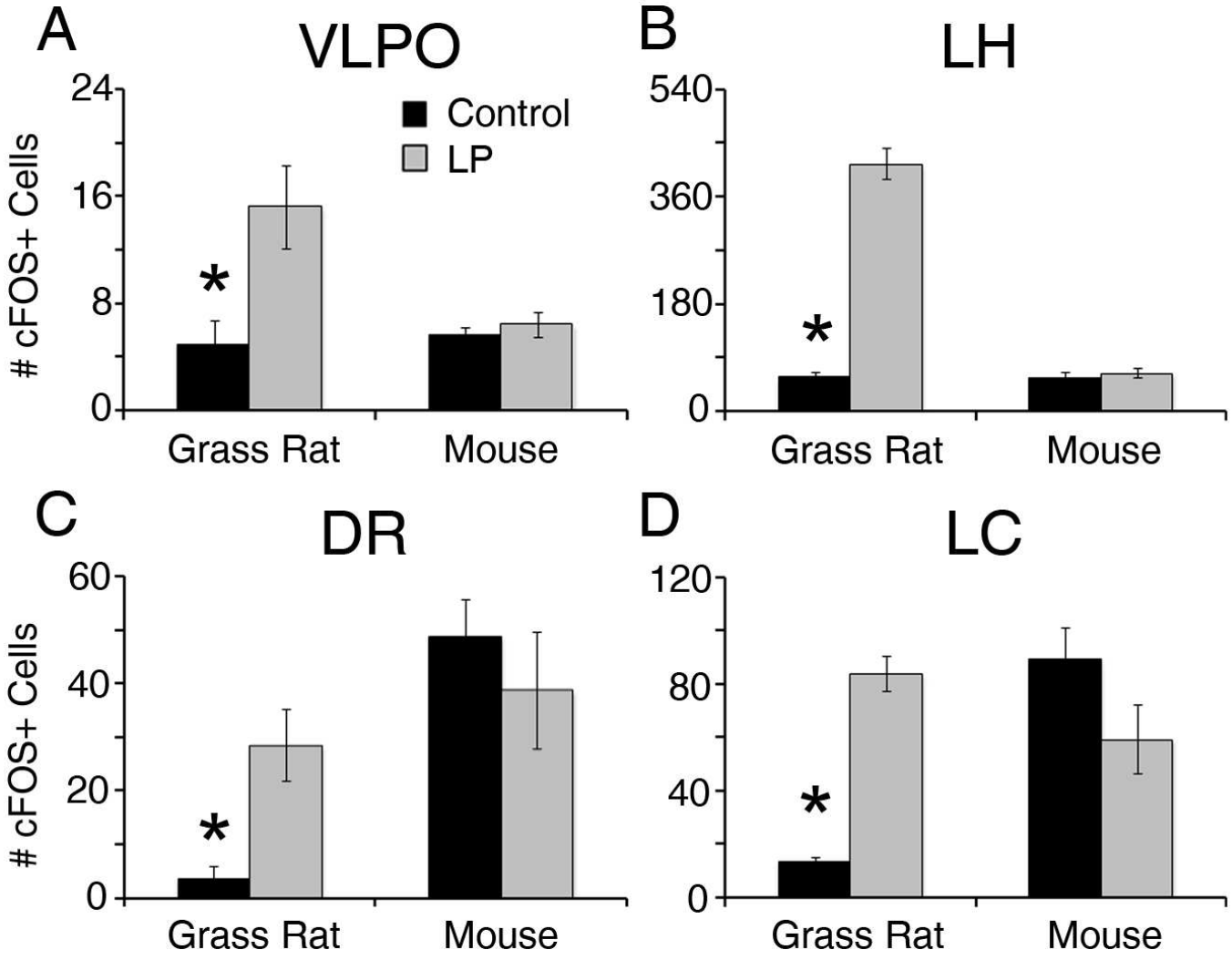


Figure 2.8. Patterns in cFOS expression in arousal/sleep-related regions. Panel A-D show the same pattern of response for within each species, respectively. In the grass rat cFOS activation is observed in the VLPO (A), LH (B), DR (C), and LC (D). No response was observed in any of the arousal/sleep-related regions in the mouse tissue. An asterisk (*) indicates significance $p < .05$.



Finally, in the VLPO (Figure 2.7), a sleep-promoting of the hypothalamus, light induced an increase in cFOS in the grass rats ($t(5)=5.87$, $p<0.01$) but had no effect in mice ($t(8)=0.74$, $p=0.481$).

DISCUSSION

In the current study we found that behavioral responses to light were clearly inverted in diurnal grass rats compared to nocturnal mice. While 1 hr of exposure to a 300 lux photic stimulus increased general activity in diurnal grass rats, it decreased activity of nocturnal mice. This result was not surprising, as it replicates our earlier findings in the same species (Shuboni et al., 2012), and the suppression of activity by light has been seen in many studies in nocturnal rodents (Butler & Silver, 2011; Morin et al., 2010; Pendergast & Yamazaki, 2011; Redlin & Mrosovsky, 1999b). It should be noted that the increase in activity observed in the grass rats was not a fear-induced startle response, as this behavior is only produced with high levels of light (>1000 lux, data not shown). However, relationships between masking and photic induction of cFOS in areas that are retino-recipient, or related to sleep/arousal, were more complicated.

cFOS expression in Retino-Recipent Regions

Retinal projections have been well described by others in both mice (Hattar et al., 2006; Mikkelsen, 1992) and grass rats (Smale & Boverhof, 1999), but our tract tracing results enabled us to confirm that retinal projections extend to the regions discussed below and that these projections do not differ substantially in grass rats and CD1 mice (Figure 2.1 A-B). When considering the cFOS responses of different retino-recipient

areas in grass rats and mice, four general patterns become apparent: (1) In one area, the SCN, grass rats and mice responded to light in the same manner. This, result is consistent with earlier data from both species and provides assurance that the fundamental parameters of the experimental manipulation (e.g. light intensities) and methods used to assess cFOS were sound. (2) In a second area, the VMH, there was no response in either species, providing assurance that light-induced changes in cFOS observed here did not reflect a simple widespread reaction to the stimulus, but can provide insight into more focused functional responses to photic stimuli that induce masking. (3) In three retinorecipient areas, the vSPZ, IGL and LHb, light induced an increase in cFOS in grass rats but had no effect in mice. Thus, there is a relationship between the increase of activity by light and photic induction of cFOS in these regions in grass, whereas these responses are dissociable in mice, suggesting that masking of behavior by light does not depend on induction of cFOS in these areas in mice, though it might in grass rats. (4) Finally, in two brain regions, the DLG and OPN, the responses seen in mice and grass rats were in opposite directions. The implications of these species differences are discussed below, but first it should be noted that in some regions the patterns of cFOS in our mice were different from those described in earlier published reports; these are highlighted in Table 2.1. Many potentially important experimental parameters vary among these studies, including light intensity, time of day at which animals were exposed to light, the duration of the light pulse and, perhaps most importantly, the strain of mouse used in these studies. To the author's knowledge, there are no reports of cFOS activation in CD1 mice after exposure to a nighttime light pulse. We chose this strain because, unlike many others, melatonin is elevated at night

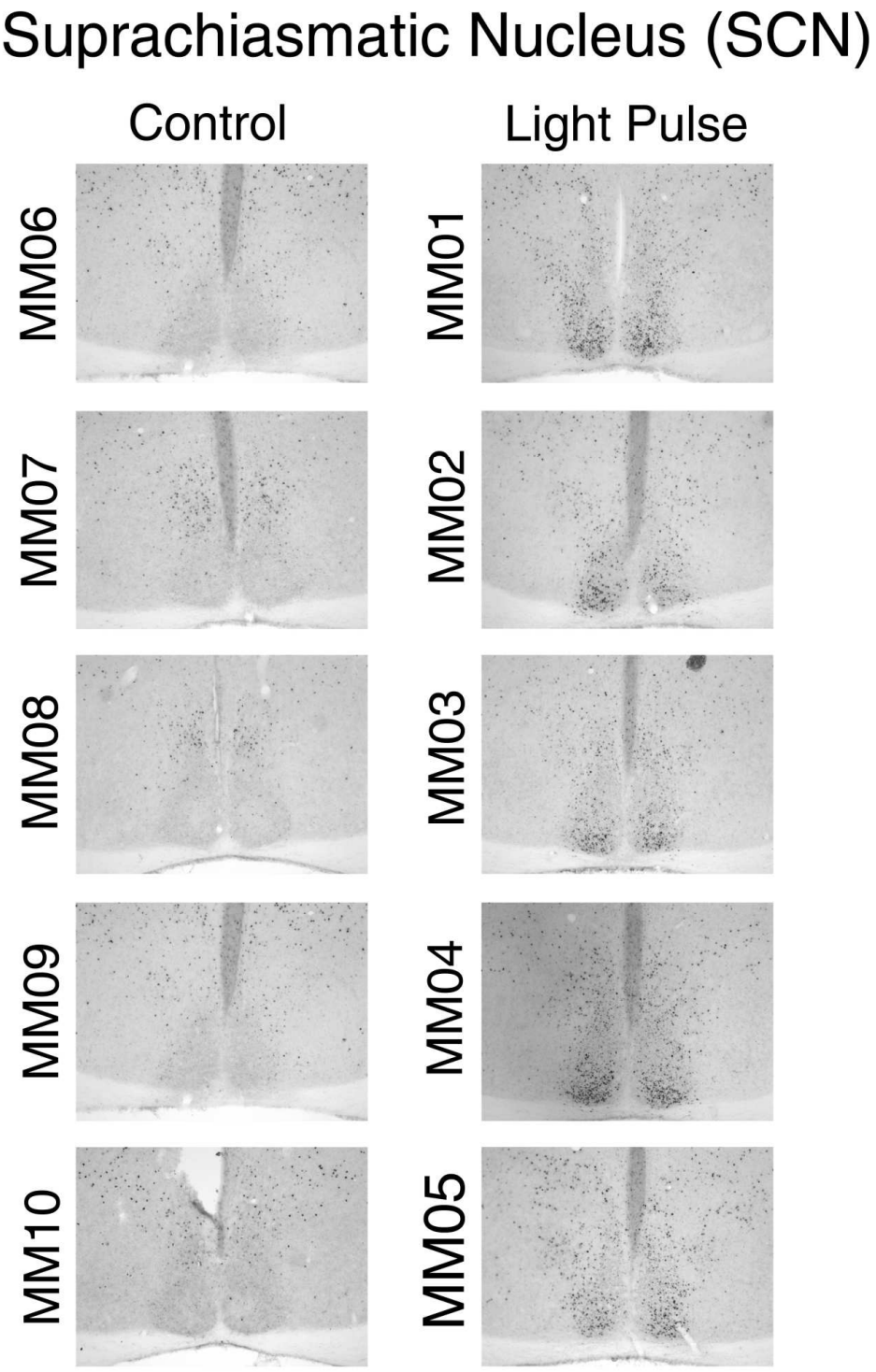
and directly suppressed by light (i.e. masked), as it is in other mammals. Neural pathways associated with the masking response of the pineal gland to light may also play a role in direct responses of some brain regions to photic stimuli. It is also possible that a light-induced decrease in melatonin could cause a change in the nature of the cFOS response to light in these regions (though the results described in Chapter 4 suggest that this is unlikely).

SCN

In the SCN, we found that both species showed a clear increase in cFOS after the light pulse (Figure 2.3, 2.4 & 2.9). This pattern of activation has been demonstrated before in both grass rats and mice pulsed with light during the dark phase of a 24 hr LD cycle as in the current study (Grass rat: Katona, Rose, & Smale, 1998; Schwartz et al., 2004; Mouse: Colwell & Foster, 1992; Masana, Benloucif, & Dubocovich, 1996), as well as when they are kept in DD and exposed to a pulse of light during the subjective night (Grass rats: Mahoney et al., 2001; Mouse: Geusz et al., 1997; Miller et al., 1999). The focus of research on induction of cFOS in the SCN by light has been on its role in phase shifting of the endogenous circadian oscillator, and there is good evidence that it does play such a role in nocturnal rodents (Honrado et al., 1996; Wollnik et al., 1995). This is likely to be the case in grass rats as well, as effects of light on the circadian system in these animals are very similar to those seen in nocturnal species (Smale et al., 2003).

The possibility that light-induced cFOS in the SCN plays a role in masking has not been examined in any species, though the question of whether the SCN itself is

Figure 2.9. Photomicrographs of the suprachiasmatic nucleus and ventral subparaventricualr zone for all mouse subjects. On the left, all control mice have low levels of cFOS in the SCN while on the right LP animals have high levels of cFOS expression. An examination if the vSPZ, reveals that control animals vary greatly as three animals have high levels of expression (MM07, MM08, & MM10) and two have lower levels (MM06 & MM09).



important for masking has been addressed in numerous studies of SCN lesioned nocturnal rodents. As noted above, one form of masking that is the same in nocturnal and diurnal species is the suppression of melatonin secretion by light at night, and this appears to depend on the SCN (Perreau-Lenz et al., 2003; Perreau-Lenz, Kalsbeek, Pevet, & Buijs, 2004; Studholme et al., 2013). The role of the SCN in masking of behavior has been directly tested in only two studies of nocturnal animals (hamsters), and these produced conflicting results (Li et al., 2005; Redlin & Mrosovsky, 1999a). The presence of behavioral rhythms in SCN-lesioned animals maintained in a 24 hr LD cycle can also indicate the maintenance of masking, but here too, the results are mixed (Table 2.2); they are also complicated by the fact that SCN lesions may cause varying degrees of damage to the optic chiasm (Mistlberger, 1992). The role of the SCN in masking thus remains unclear in nocturnal species, and there are no data on this issue in diurnal species (however, see Table 2.2 and Chapter 3).

vSPZ

The vSPZ is one of the three retino-recipient regions in which light produced a robust increase in cFOS expression in grass rats but had no effect in mice (Figure 2.10). The induction of cFOS in this region by light has been seen previously in grass rats (Mahoney et al., 2001), as well as nocturnal rodents, including hamsters, rats, and mice (Brooks et al., 2011; LeGates et al., 2012; Todd, Gall, Weiner, & Blumberg, 2012; Y. Zhang et al., 1993). Thus, the lack of a response in our mice is somewhat surprising. It is not likely to be due to technical difficulties, as in these same animals we were able to see clear light-induced masking in their SCN-lesioned hamsters might be attributable

to the destruction of retinal input to that area. In rats, lesions of the vSPZ that leave the SCN intact eliminate increases and decreases in cFOS in other retino-recipient brain regions (Figure 2.9). As noted above, the differences among studies might be attributable to the fact that our strain of mice was not the same as those used in the two earlier studies (Brooks et al., 2011; LeGates et al., 2012). In any case, the current results suggest that in these mice, the masking of activity by a photic stimulus can occur in the absence of a cFOS response of vSPZ neurons to that stimulus.

The question of whether the presence of a cFOS response of cells in the vSPZ reflects a role that this region may play in masking is more complex. It is an interesting candidate region because it receives input from both the retina (Figure 2.1A-B; (Hattar et al., 2006; Johnson, Morin, & Moore, 1988; Smale & Boverhof, 1999), and the SCN (Kriegsfeld, Leak, Yackulic, LeSauter, & Silver, 2004; Morin, Goodless-Sanchez, Smale, & Moore, 1994; Schwartz, Urbanski, Nunez, & Smale, 2011), and it projects to many of the same areas as the SCN (Canteras, Ribeiro-Barbosa, Goto, Cipolla-Neto, & Swanson, 2011; Schwartz et al., 2011; Watts, Swanson, & Sanchez-Watts, 1987). It is thus well positioned to integrate direct photic signals from the retina with circadian signals, and to modulate the same functions (e.g. activity) that are regulated by the SCN. There is some evidence that the vSPZ may contribute to diurnality of the grass rat circadian system, as it exhibits rhythms in cFOS that are very different in from those seen in nocturnal lab rats (Nunez, Bult, McElhinny, & Smale, 1999) and that persist in DD (Schwartz et al., 2004). Lesions in this area also lead to a reduction in the ratio of activity during subjective day relative to night in these animals and altered

Table 2.2. Literature review of masking in12:12 LD condition for animals with suprachiasmatic nucleus lesions. (†) indicates studies that observed extreme damage to the optic chiasm. (*) indicates studies that use lack of rhythms in LD as a criteria for good SCN lesion.

Author	Species	Strain	Variable	Masking
Easton, 2004	Mice	C57Bl/6J	GA, EEG, BT	N*
Tong, 2013	Mice	ddY	HR and BT	N
Stephan & Zucker, 1972	Rat	Sprague-Dawley	Drinking and Wheel running	N†
Coindet, 1975	Rat		EEG	N
Mistlberger, 1983	Rat	Sprague-Dawley	EEG	**Y**
Liu, 2012	Rat	Sprague-Dawley	EEG	N
Ibuka & Kawamura, 1975	Rat	Albino	EEG	N
Ibuka, 1977	Rat	Wistar	EEG	N
Aguilar-Roblero, 1986	Rat	Wistar	Drinking	N
Scheer et al., 2001	Rat	Wistar	Drinking, HR, GA	N*

Table 2.2. (cont'd)

Author	Species	Strain	Variable	Masking
Amir et al., 2004	Rat	Wistar	Wheel Running	N*
Zhang et al., 2004	Rat	Wistar	GA & BT	**Y**
Hu et al. 2007	Rat	Wistar	GA	N
Angeles-Castellanos, 2010	Rat	Wistar	GA & BT	N*
Warren, 1994	Rat	Long Evans	GA, HR, BT	N
Wachulec, 1997	Rat	Long Evans	Drinking, BT, GA	**3Y 5N†**
Mistlberger, 1992	Hamster	Golden Syrian	Wheel running	**10Y 2N†**
DeCoursey, 1997	Ground Squirrel		Wheel running	**Y**
Fuller, 1981	Squirrel Monkey		Drinking and BT	**Y**
Sato & Kawamura, 1984	Chipmunk	Siberian	Wheel running	**1Y 3N**

† Extensive damage to the Optic Chiasm

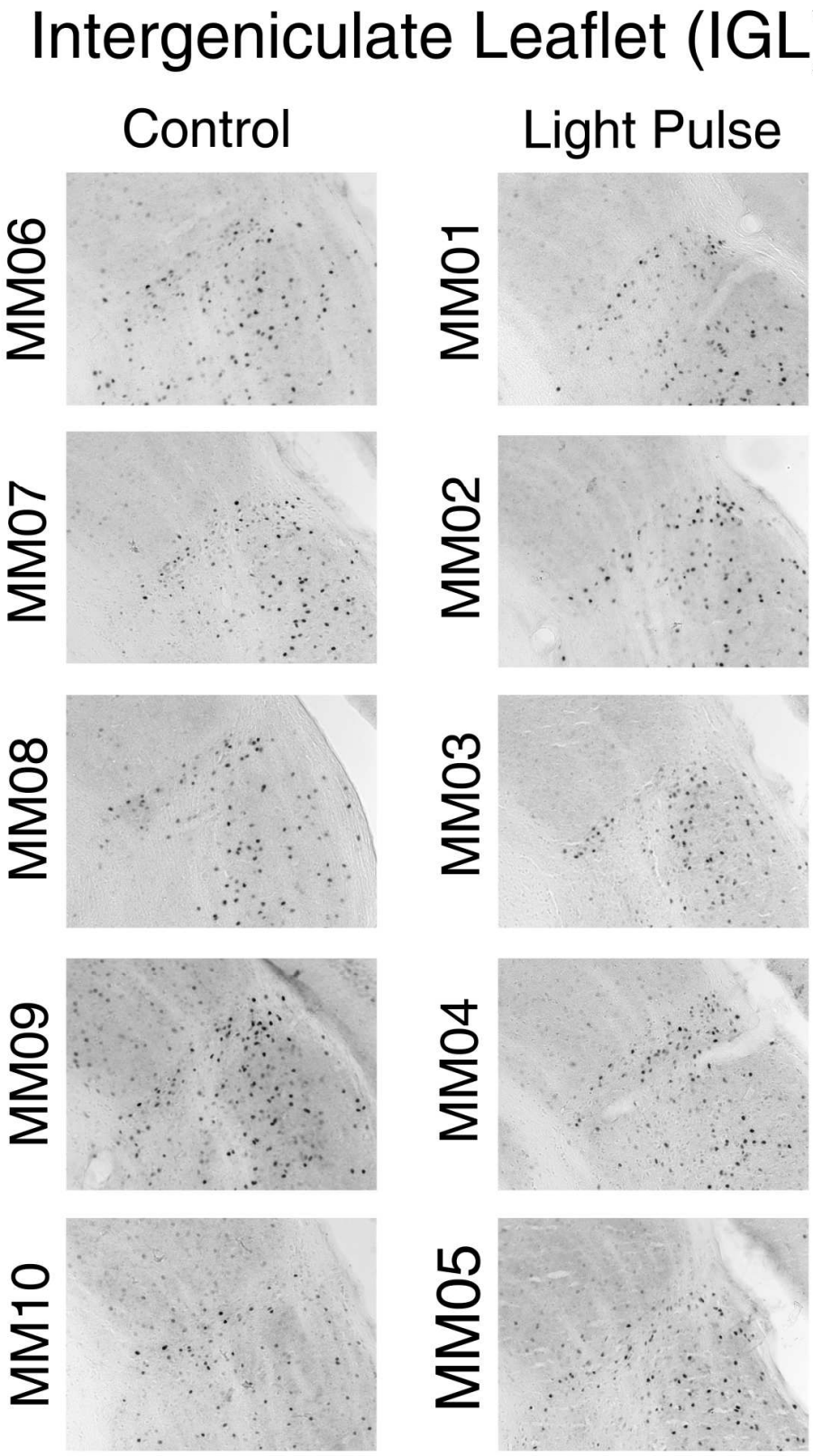
* Used lack if rhythm as criteria for good lesion

reentrainment rates (Schwartz, Nunez, & Smale, 2009). The role of the vSPZ in masking has not been directly tested, though Li et al. (2005) suggested that the loss of endogenous rhythms in activity, suggesting that this region it is an essential output pathway for the circadian regulation of activity. However, these lesioned animals do exhibit rhythms in LD conditions (Abrahamson & Moore, 2006; Cipolla-Neto et al., 1995; Lu et al., 2001; Moore & Danchenko, 2002), suggesting that the vSPZ is not required for the masking response of activity to light in these animals. Finally in Chapter 3, I describe evidence that direct masking to light pulses is maintained in grass rats with ablated vSPZ, suggesting that although cells in this region respond in an acute manner to light (Figure 2.3) they are not necessary for masking in grass rats.

IGL

The second of the three retino-recipient regions in which light produced an increase in cFOS in grass rats but had no effect in mice is the IGL (Figure 2.10). This result suggests that mechanisms associated with light responsiveness of the IGL of grass rats are similar to those of another diurnal species, the *Octodon degus*, as, when those animals are held in DD 1 hr of light at CT16 can stimulate an increase in cFOS in the IGL (Krajnak et al., 1997). There are far more studies of this issue in nocturnal rodents, and here the story are somewhat more complicated. Although there is good evidence that light induces cFOS in the hamster IGL, the published data on rats and mice are inconsistent (Table 2.2). Several studies have shown that light can induce cFOS in the rat IGL but most of these have looked at least 2 hrs after exposure to the

Figure 2.10. Photomicrographs of the intergeniculate leaflet for all mouse subjects. Both control (Left) and LP (Right) animals have high levels of cFOS expression.



light began (Aronin et al., 1990; Janik & Mrosovsky, 1992; Peters et al., 1996; Rusak et al., 1990; Todd et al., 2012), and in one study in which rats were sacrificed just 30 and 90 minutes after lights were turned on there was no increase in cFOS (Park et al., 1993). In their review of data on photic induction of cFOS, (Kornhauser, Mayo, & Takahashi, 1996) suggest that in rats an increase in cFOS in the IGL can only be detected two or more hours after the light stimulus begins. Interestingly, Juhl et al. (2007) found that while 90 minutes of light at ZT14 or ZT19 did not induce a change in overall cFOS in the rat IGL, it did elevate cFOS within the subset of cells that contain enkephlin; no change was seen in neuropeptide Y-containing calls. One important question is whether light induces cFOS in the same subpopulations of IGL cells in diurnal grass rats as in nocturnal lab rats. Perhaps, for example, cFOS is induced in projection neurons in lab rats and in inhibitory interneurons in grass rats. It should be noted that the experiments described above were conducted on Sprague-Dawley and Wistar rats, and that a more recent study using Fischer 344 rats did find a significant rise in cFOS in the IGL after only 1 hr of exposure to light at midday and midnight (Prichard et al., 2002). In the case of mice, there are two studies that show an increase in cFOS in the IGL (Delogu et al., 2012; Lupi et al., 2012) and one in which the animals did not respond to a light pulse (Lupi et al., 1999). Again, the strain of the mice might account for the differing results, as C57/BL/6J and C3H/He mice responded differently even when kept in the same laboratory and exposed to the same irradiance levels at the same times of day (Lupi et al., 1999; Lupi et al., 2012). Taken together, the published data on light-induced cFOS in the IGL of rats and mice suggest that the absence of

light-induced cFOS in our CD1 mice might reflect a strain difference, and/or that induction of cFOS takes longer than 1 hr in these animals.

The patterns of responsiveness of grass rats and mice seen in the current study raise the question of whether the IGL might contribute to differences in their behavioral responses to that light (i.e. masking). Several kinds of data suggest that the IGL might play a role in masking. As is the case for the SCN and vSPZ, the retinal input to the IGL comes from ipRGCs in mice (Hattar et al., 2006), and these cells play an important role in masking (Mrosovsky & Hattar, 2003). Lesion studies have also produced interesting results relating the IGL to masking, in both nocturnal and diurnal animals. In nocturnal rodents, masking is retained under LD conditions after the IGL is lesioned (Cipolla-Neto et al., 1995; Edelstein & Amir, 1999; Redlin et al., 1999) and in hamsters the masking response of wheel running to light is actually increased after the IGL has been destroyed (Edelstein & Amir, 1999). The most striking effect of IGL lesions on masking, however, is that seen in diurnal grass rats, as Gall et al. (2013) showed that destruction of the IGL actually reversed the direction of the masking response, causing animals to respond to light with a decrease, rather than an increase, in activity. Results from lesion studies, as well as the cFOS response shown here, suggest that the IGL plays an important role in the ability of grass rats to sustain a masking response typical of diurnal animals.

LHb

The third retino-recipient brain region in which light increased cFOS in the grass rat but had no effect in mice is the LHb. Only one other study that we are aware of has

directly assessed effects of light on cFOS in this region in nocturnal mice, and that study found a large stimulatory effect (LeGates et al., 2012). On the other hand, in an LD cycle, cFOS in this region is higher during the night than during the day in nocturnal rodents, including mice, rats and hamsters (Tavakoli-Nezhad & Schwartz, 2006). It should also be noted that cFOS patterns suggest that the habenula is activated in response to activity (Engber et al., 1998; Paul, Indic, & Schwartz, 2011; Tavakoli-Nezhad & Schwartz, 2006), and stress (Chastrette, Pfaff, & Gibbs, 1991; Lehner et al., 2004). Perhaps differences in these parameters could account for the differences between our results with mice and those described by LeGates et al. (2012).

We were particularly interested in the habenula because its direct access to light information from the retina (Hattar et al., 2006) and its projections to arousal-inducing areas of the brain (Aizawa, Kobayashi, Tanaka, Fukai, & Okamoto, 2012; Andres, von During, & Veh, 1999; Herkenham & Nauta, 1979) putting it in a good position to mediate masking. *In vivo* electrophysiological studies in rats have shown that neurons in the habenula, particularly its lateral subregion, respond to acute presentation of light, and *in vitro* recordings reveal a circadian rhythm in its firing rate (Zhao & Rusak, 2005). Though it is not yet clear whether the LHb contributes to masking, or to species differences in its manifestation, its responsiveness to light in grass rats suggest that it is a region that should be explored further in efforts to better understand these issues.

DLG

The dorsal lateral geniculate (DLG) of the thalamus is one of the two brain regions in which light induced an increase in cFOS in the grass rat and a decrease in

mice (Figure 2.3). Although a light-induced decrease in cFOS in the habenula has not been reported before, it should be noted that (Prichard et al., 2002) saw no effect of a 2 hr pulse of light at midnight on cFOS in the DLG of rats. In contrast to these nocturnal rodents, grass rats exhibited a robust increase in response to light, as has been reported in two other diurnal species, treeshrews (Poveda & Kretz, 2009) and Mongolian gerbils (Fite, Wu, & Bellemer, 2005). The DLG is an important part of the primary visual system (Hendrickson, Wilson, & Ogren, 1978; Hoffmann, Stone, & Sherman, 1972), but it also plays a role in the masking behavior of mice, as lesions in this area enhance the inhibitory effect of light on activity in these animals (Edelstein & Mrosovsky, 2001). The possibility that the DLG plays a role in masking in grass rats and other diurnal species has not yet been examined, but the data here suggest the possibility of interesting and important differences in the role that it might play in nocturnal and diurnal species.

OPN

The second region in which light increased cFOS in the grass rat and decreased it in mice was the OPN (Figure 2.3). In this case, the control mice appeared to have very high levels of cFOS, and light led to a decrease of more than 50% (Figure 2.6). This was surprising to us because an earlier study, by Prichard et al. (2002) had found that a 2 hr pulse of light at midnight can induce a significant increase in cFOS within the OPN of nocturnal lab rats. However, when light exposure occurred during the day it did not have an effect. Differences in the time of sampling in these studies may, therefore,

account for the apparent inconsistency, although it is possible that it reflect differences between lab rats and mice.

The focus of research on the OPN has been on the role that it plays in the papillary light reflex (Allen, Brown, & Lucas, 2011) but the same characteristics that enable it to do that are ones that would enable it to contribute to masking. It receives substantial input from melanopsin-containing cells in the retina (Hattar et al., 2006), contains neurons that are capable of coding illumination levels (Szkudlarek, Orlowska, & Lewandowski, 2012) and it projects to at least one region that appears to play a role in masking, the IGL (Morin & Blanchard, 1995). Additionally, removal of the OPN interferes with masking of REM sleep by darkness in albino rats (Miller et al., 1999). However, the role of this region in masking of general activity by photic stimuli has not been examined in either nocturnal or diurnal species.

cFOS expression in Arousal/Sleep-Related Regions

When considering the cFOS responses to light in the 4 arousal/sleep-related areas examined here, only one general pattern becomes apparent: in all of them light induced an increase in cFOS in grass rats but had no effect in mice. The implications of these species differences are discussed below, but first it should be noted that in this case there are few published data with which to compare our results.

VLPO

In the *VLPO*, a region of the hypothalamus known to promote sleep in some species (Lu et al., 2001; Sherin, Shiromani, McCarley, & Saper, 1996), we found that

the same light that stimulated activity actually increased cFOS in grass rats (Figure 2.7). The reasons for this paradoxical response are not clear, but one possibility involves the internal circuitry of the VLPO, where the subset of cells that actually stimulate sleep in nocturnal rodents are known to contain galanin, and cFOS is elevated in them during sleep (Gaus, Strecker, Tate, Parker, & Saper, 2002). Thus, it may be that in grass rats, but not mice, light activates inhibitory interneurons that suppress galanin-containing cells in the VLPO, leading to a reduction in sleep.

In mice, we found that while light decreased activity it did not affect cFOS in the VLPO. Previous experiments examining light-induced cFOS in this area in mice have produced results that are somewhat contradictory. Lupi et al. (2008), using RT-PCR, saw a significant light-induced increase in *fos* mRNA when mice were pulsed for 1 hr with light. However, Tsai et al. (2009) saw no effect of light on overall levels of cFOS in this region but they did when they focused specifically on the sleep-promoting galanin-positive cells there. It should be noted that the VLPO is innervated by fibers originating in the retina in grass rats and mice (Figure 2.1), though there is some evidence that the density of that retinal input is higher in nocturnal lab rats than grass rats (Nunez, unpublished results); this does not appear to be the case in mice. Many of these fibers are known in mice to come from melanopsin-containing retinal ganglion cells (Gooley et al., 2003). Several investigators have suggested that this pathway may play a role in masking (Lupi, Oster, Thompson, and Foster, 2008; Redlin, 2001) but no on has directly examined the region's role in the masking response.

LH, DR, LC

The LH, DR and LC are of interest in understanding the neural mechanisms of masking as each plays an important role in induction and maintenance of arousal (Mistlberger, Antle, Kilduff, & Jones, 2003; Sara & Bouret, 2012; Tsujino & Sakurai, 2013). In all three of these brain regions, grass rats were responsive to light, while mice were not. The absence of an effect of light on cFOS in arousal-inducing structures of mice has been seen by others (Mendoza, Clesse, Pevet, & Challet, 2010), and is likely not a simple reflection of the rate of decay in the protein. Light-induced cFOS has been seen previously in the LH and DR of grass rats (Adidharma, Leach, & Yan, 2012), as well as the DR of another diurnal murid rodent, the Mongolian gerbil (Fite et al., 2005). Interestingly, the retina projects directly to the LH of grass rats (Figure 2.1), and to the DR of Mongolian gerbils (Fite, Birkett, Smith, Janusonis, & McLaughlin, 2003). It is tempting to speculate that these pathways play a role in the induction of cFOS and masking in these diurnal species.

Summary and Conclusions

The behavioral response of nocturnal and diurnal rodents to light exposure are polar opposites, with nocturnal species decreasing activity and diurnal species increasing activity after a light pulse. Most of the brain regions we examined showed a very different response to light in mice and rats. In some cases, the response of the mice was very different than those observed in other nocturnal rodents. Above we explored several possible explanations for the discrepancies, including age, duration of exposure, other FOS-inducing stimuli, and most importantly strain differences. Although those regions varied, we did observe a clear and similar uni-direction response in the

SCN in grass rats and mice, indicating that we replicated a well-established pattern in our animals. Therefore, we believe that these findings demonstrate a interesting differences between nocturnal mice and diurnal grass rats in the responsiveness of other regions to light that will provide the framework for targeting regions in future attempts to better understand the masking response to light and how the system varies between species that occupy different temporal niches.

CHAPTER 3

Ventral subparaventricular zone lesions and light-induced masking behavior in the diurnal rodent, *Arvicanthis niloticus*

ABSTRACT

The ventral subparaventricular zone (vSPZ) is a region of the brain that plays a major role in regulation of circadian rhythms in sleep and activity. Animals most active during the day (diurnal animals), display different patterns of cellular activation and firing rates in the vSPZ when compared to animals active during the night, (nocturnal animals). Masking behavior, an acute behavioral response to an external stimulus, also differs between diurnal and nocturnal animals, with light inhibiting activity in nocturnal animals and stimulating it in diurnal ones. Little is known about the neural structures that mediate masking, but the vSPZ is a promising candidate as it receives direct retinal input and it responds to light with a rapid increase in cFOS. Here we tested the effects of chemical ablation of the vSPZ on the ability of animals to exhibit acute responses to the presentation of light and to maintain circadian rhythmicity under different lighting conditions. The vSPZ was successfully abolished in 4 animals; in 2 of these, the SCN was also virtually destroyed. Two of the 4 animals retained the ability to respond to light pulses with an immediate increase in activity; 3 of these 4 animals with no vSPZ showed masking in the context of a 3.5:3.5 ultradian light:dark (LD) cycle. In a 12:12 LD cycle, the same three animals had clear daily rhythms, but when placed into constant darkness and then constant light, only one of these animals displayed a significant circadian rhythm. These data establish that the vSPZ is not necessary for the masking response to light and suggest that the grass rat vSPZ may contribute to circadian

regulation. Additionally, in the grass rat the NMA is capable of destroying the SCN, a structure generally believed to be resistant to this chemotoxin.

INTRODUCTION

Light is a powerful daily signal, indicating the progression of time across the 24 hr day. In mammals, light influences the daily pattern of activity through two important mechanisms, entrainment of circadian rhythms and masking, that function in a synergistic manner to promote the appropriate behavior at the correct time-of-day (Mrosovsky & Thompson, 2008). The immediate change in behavior, such as general activity, after the acute presentation of light is called the masking response (Aschoff, 1960). The direction of this response is dependent on the temporal niche an animal occupies. Among diurnal species light triggers an increase in activity while for nocturnal species the opposite is observed (Mrosovsky, 1999). This difference has long been established (Aschoff & Vongoetz, 1989; Borbely & Huston, 1974), however, until recently the importance of masking in the evolution of diurnality has received little attention (Chiesa, Aguzzi, Garcia, Sarda, & de la Iglesia, 2010; Cohen et al., 2010; Mrosovsky & Hattar, 2005; Rotics et al., 2011). Understanding where and how the brain responds to a masking pulse of light would provide greater insight into the changes that must occur for an organism to transition from nocturnal to diurnal pattern of behavior. However, almost nothing is known about the neural mechanisms of masking in diurnal species.

In nocturnal species, investigations into the brain structures important for the masking response to light have established two facts. First, masking is dependent on the signal generated by cells within the retina that contain melanopsin (ipRGCs) after they are exposed to light (Hatori et al., 2008; Mrosovsky & Hattar, 2003; Panda et al., 2003). This suggests that areas innervated by ipRGCs could play an important role in

the regulation of masking. ipRGC projections extend to many regions that receive retinal input outside of the primary visual pathway, including the suprachiasmatic nucleus (SCN), olivary pretectal nucleus (OPN), intergeniculate leaflet (IGL), and the ventral subparaventricular zone (vSPZ; Hattar et al., 2006). Second, lesions of no one region have thus far clearly eliminated masking (Redlin, 2001). The destruction of the IGL enhances the inhibitory effects of light on activity in hamsters (Redlin & Mrosovsky, 1999b), while removal of the OPN attenuates the masking of REM by darkness in albino rats (Miller et al., 1999). Lesions of the SCN have produced mixed results, with both positive (Li et al., 2005) and negative (Redlin & Mrosovsky, 1999a) findings. One explanation posed by Li et al. (2005) for the discrepancy, is that extensive damage to the retinal fibers innervating the vSPZ caused the loss of the masking response in their animals. The vSPZ could, therefore, play an important role in masking.

The vSPZ is a region of the brain found dorsal to the SCN that receives major input from the SCN, and that projects to many of the same areas as the SCN, in both nocturnal (Kriegsfeld et al., 2004; Morin et al., 1994) and diurnal (Schwartz et al., 2011) species. The vSPZ is of interest in the understanding of diurnality for a variety of reasons. Diurnal animal have different daily patterns of cellular activation (Nunez et al., 1999; Schwartz et al., 2004) and firing rates (Sato & Kawamura, 1984) in the vSPZ when compared to nocturnal animals. The area is known for playing a major role in regulation of circadian rhythms of sleep and activity. Chemotoxic and electrolytic lesions of the vSPZ abolish circadian activity rhythms of nocturnal rodents when they are kept in constant darkness (Abrahamson & Moore, 2006; Cipolla-Neto et al., 1995; Lu et al., 2001; Moore & Danchenko, 2002). Partial lesions of the vSPZ of grass rats do not have

the same dramatic effect, but there is a correlation between the size of the lesion and rhythm strength (Schwartz et al., 2009). Beyond the region's role in circadian rhythms, the vSPZ may also impact the expression of daily activity by influencing masking. As mentioned above, the vSPZ receives input from melanopsin cells in mice, and data described in Chapter 2 revealed that the region has a niche dependent cFOS response to light exposure. Additionally, grass rats with partial lesions in the area have bouts of arrhythmia in a 12:12 light:dark (LD) cycle (Schwartz et al., 2009), which suggests a diminished masking response to photic stimuli. Taken together, the data suggest that the vSPZ is an ideal target area for the investigation of the neural mechanisms of masking.

Here we used the diurnal rodent, *Arvicanthis niloticus* (Blanchong et al., 1999), to test the hypothesis that the vSPZ is a part of the neural circuitry that mediates masking and promotes the differences in the masking response associated with an animal's temporal niche. We did this by determining whether lesions of the region affect the masking of general activity by acute presentations of light and in the context of an ultradian LD cycle. We also asked how circadian activity rhythms of vSPZ-lesioned animals are affected by different light conditions.

METHODS

Animals

Thirty-five adult female *Arvicanthis niloticus* (grass rats) were obtained from the breeding colony at Michigan State University. All animals were singly housed in Plexiglas cages (34x28x17cm) and given food (PMI Nutrition Prolab RMH 2000,

Brentwood, MO) and water *ad libitum* for the duration of the study. The facilities were maintained at a temperature ranging between 20-25°C with a 12:12 LD schedule. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University and were in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

Experimental Procedures

At the onset of the experiment, animals were transferred into a room with uniform fluorescent lighting, which exposed all animals to 300 lux. Each animal was housed in an individual cage equipped with infrared detectors (IRs, Visonic Tel Aviv, Israel) attached to the cage top. Activity was recorded in 5-minute bins using the VitalView 4.0 Software (Minimiter, Bend, OR) on a computer located in an adjacent room. Animals were monitored for at least 10 days in 12:12 LD conditions prior to the surgery. Animals then were all assigned to either a vSPZ lesioned group (SPZx) or a sham control group. After the surgeries (described below) animals were allowed to recover for a minimum of two weeks.

Animals were first tested for their masking response to light using a procedure outlined in Shuboni et al. (2012). In brief, animals were exposed to 1 hr light pulses (LPs) at three different circadian times (CTs, CT0 = activity onset) during the subjective night (CT14, CT18, CT22) and once during the subjective day (CT6). This was accomplished by releasing animals into constant darkness for two days, once every four days; animals were exposed 1 h pulses of light on the second of these two days; the order of the pulses was randomly selected. Additionally, animals were exposed to a 7 h

ultradian light:dark cycle (3.5:3.5 LD) for nine days, during which time they exhibited free-running circadian rhythms; analysis of the final seven days in these lighting conditions reveals effects of masking independent of the circadian influence on activity. Finally, some features of the circadian regulation of activity were examined by placing animals into 12:12 LD for two weeks, constant darkness (DD) for two weeks, and constant light (LL) for three weeks. At the completion of the study, animals were sacrificed and brains collected for histological assessment of the lesions.

Surgical Procedures

Thirty animals underwent the operation to bilaterally lesion the vSPZs with NMA and five experienced the same procedure but were only injected with the control vehicle (SHAM). In brief, grass rats were first anesthetized using isoflorane (2.5-5%, Abbott Laboratories, IL, USA) and then injected s.c. with Ketoprofen (5 mg/kg of body weight). Prior to the surgery, the surface of the head was shaved and sterilized with betadine. Animals were then secured in a steretaxic apparatus (Stoelting Co.; Wood Dale, IL) with the tooth bar set to -4.5 mm and then injected s.c. on the top of the head with lidocaine (0.03 mL). A 2 cm incision was made on the scalp to expose the surface of the skull and, using a surgical drill, two small 2 mm holes were made at the appropriate coordinates. Injection coordinates were +0.16 mm anterior and ±0.05 mm lateral to bregma and 6 mm ventral to the dura. Injections were made using a 0.5 μL Hamilton syringe (Hamilton, Reno, Nevada, USA) filled with either 0.3M NMA (Sigma, St. Louis, Missouri, USA) in 0.2M saline, or the vehicle control. All animals were injected with 180 nL of solution per side using a manual stereotaxic microinjector (Stoelting), injections

were given over 5 a minute period (36 nL/min) and the needle was left for 1 minute before being withdrawn. At the conclusion of the surgery, the incision was closed with autoclips and animals were injected with sterile saline (2 cc, 0.9% NaCl, s.c.). For the next 2 days, all animals received the analgesic Melaxicam (0.1 mg/kg of body weight) orally in apple every 24hrs.

Assessment of the Lesions

At the completion of the experimental procedures, all animals were transcardially perfused at ZT2 as described in Schwartz et al. (2009). In brief, animals were deeply anesthetized using an i.p. injection of sodium penabarital (400mg/kg). The animals were then transcardially perfused with 0.1M phosphate buffer saline (PBS, pH 7.2) and then a 4% paraformaldehyde solution in PBS, also containing 0.075M lysine and 0.01M sodium periodate (PLP, Sigma-Aldrich, St. Louis, MO, USA). The brains were post-fixed for an additional 4 hr in the PLP solution; they were then transferred into sucrose for a minimum of 24 hr, after which the brains were cut at 30 µm along a coronal plane using a freezing-microtome. These sections were divided into three series and stored in cyroprotectant at -20℃ until further processing. One series was mounted onto gelatin-coated slides, stained for Nissl using thionin, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA). To delineate the extent of the lesions, two experimenters independently evaluated the tissue. They examined 5 sections using a light microscope (Leitz, Wetzlar, Germany) equipped with a drawing tube at 5x magnification. Both experimenters drew the lesioned area for one section rostral to the SCN, 3 sections that included the SCN (rostral, middle, and caudal representatives),

and another section caudal to the SCN. The drawings were then compared between the experimenters and each lesion was classified as bilateral hit, unilateral hit, or miss.

Data Analysis

To analyze masking, we converted the VitalView data into Microsoft excel spreadsheets. The degree of masking during the light pulses was examined by comparing the total activity of the animal during the hour of the pulse to the same hour on the previous day. A three-way mixed ANOVA was used to assess within-subjects effects of lighting condition (control and LP) and time (CT6, CT14, CT18, CT22), and the between-subjects effects of surgical condition (sham, bilateral hit, unilateral hit, and miss). Masking behavior in the context of the ultradian LD cycle was examined by comparing the total amount of activity during all the light periods to the sum of activity during all the dark periods. A two-way mixed ANOVA was used to assess within-subjects effects of lighting condition (light and dark) and the between-subjects effects of surgical condition (sham, bilateral hit, unilateral hit, and miss). Additionally, Chi-squared periodograms for all the animals were constructed for the last seven days of the ultradian LD cycle using ClockLab (Actimetrics, Wilmette, IL, USA). This analysis distinguishes significant rhythms from random fluctuations in the data (Sokolove & Bushell, 1978).

To analyze the circadian rhythms data, we converted our Vitalview files into actograms using ClockLab (Actimetrics, Wilmette, IL, USA). We also analyzed the last week in LD, DD, and LL using Chi-squared periodograms to identify circadian rhythms of the animals. Then determined onset and offset of activity and the period of the

rhythm, tau (τ) with the program, ClockLab identifies these points independently, however, the experimenter also visually inspected these points prior to analysis, to identify and manually corrected any errors (Schrader, Walaszczyk, & Smale, 2009). From the onsets and offsets we also calculated the duration of the active period, alpha (α), and inactive period (ρ), and an alpha/rho ratio was determined from those values. A two-way mixed ANOVA was used to assess effects of lighting condition (LL and DD, within subjects) and surgical condition (sham, bilateral hit, unilateral hit, and miss, between-subjects) on τ and the alpha/rho ratio. All analyses were further examined if significant interaction or main effects were present using one-way ANOVAs and t-test. Significant differences were ascribed when $p < 0.05$.

RESULTS

Histology

Though needle tracts were observed in the brains of sham animals (Figure 3.1; n=5), their SCN and vSPZ were intact (Figure 3.1). In 5 animals the toxin failed to damage the SCN or vSPZ on either side of the brain ("miss", Figure 3.2), and in 3 animals, damage to these structures was unilateral ("unilateral hit"). In these animals, the lesioned areas showed a dramatic reduction in the number of neurons (Figure 3.2). Finally, we had four successful bilateral lesions that destroyed the entire vSPZ (Figure 3.3). In all of these animals (referred to as "lesion") the retrochiasmatic area was also completely destroyed, one also sustained unilateral damage to the supraoptic nucleus and in another the caudal PVN was partially destroyed. Finally, two animals in the

bilateral hit group had extensive damage to the SCN with only small fragments of the nuclei remaining.

Masking

The mixed three-way ANOVA used to assess effects of masking pulses of light on activity violated the assumption of sphericity. We therefore analyzed each of the 4 groups individually (i.e. sham, miss, unilaterally hit, and bilateral hit), using within-subjects two-way ANOVAs. Unilaterally lesioned and sham groups continued to violate assumptions of sphericity. In the miss group there were no main effects of either lighting (F[1, 5] = 2.747, p = 0.158) or time (F[3, 15] = 1.249, p = 0.327), but there was an interaction (F[3, 15] = 8.644, p < 0.014). Among animals in the bilateral hit group there was a near-significant main effect of lighting condition, with activity levels tending to be higher during the light (F[1, 3] = 9.282, p < 0.056). To better understand the presence of masking in our animals, we looked at each individual's masking response at all time-points. Miss animals all had higher levels of activity when pulsed with light (Figure 3.4). However, this was not the case in unilateral hit (Figure 3.4) or sham animals (Figure 3.5), as one animal in each group showed no response to the light. Examination of activity of bilaterally lesioned animas also reveals a great deal of variation (Figure 3.6). Although all animals appeared to respond to light overall, this was not the case for each animal at one or more of the time points examined; the times at which activity was not higher during the light pulse than the control pulse varied from animal to animal. However, 12 of the 16 total cases had increased in activity during the light periods and only for 3 cases was there no change or a decrease in activity.

Figure 3.1. Photomicrographs of Nissl-stained tissue in all SHAM lesioned animals. Sections were selected to represent one section before the vSPZ, three spanning the rostral-caudal extent of the regions and one section after. No damage is observed in the tissue with the exception of needle marks.

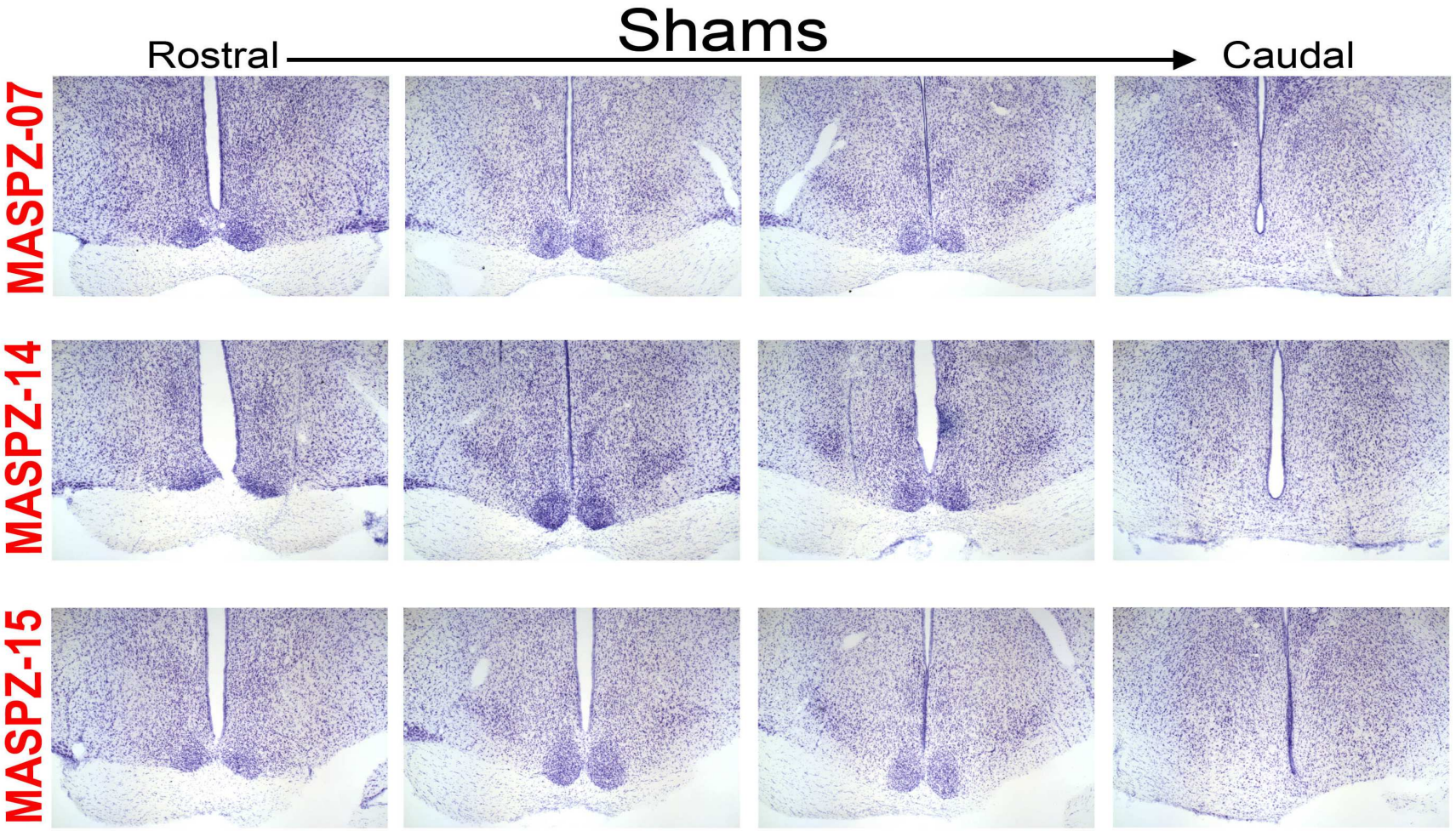


Figure 3.1. (cont'd)

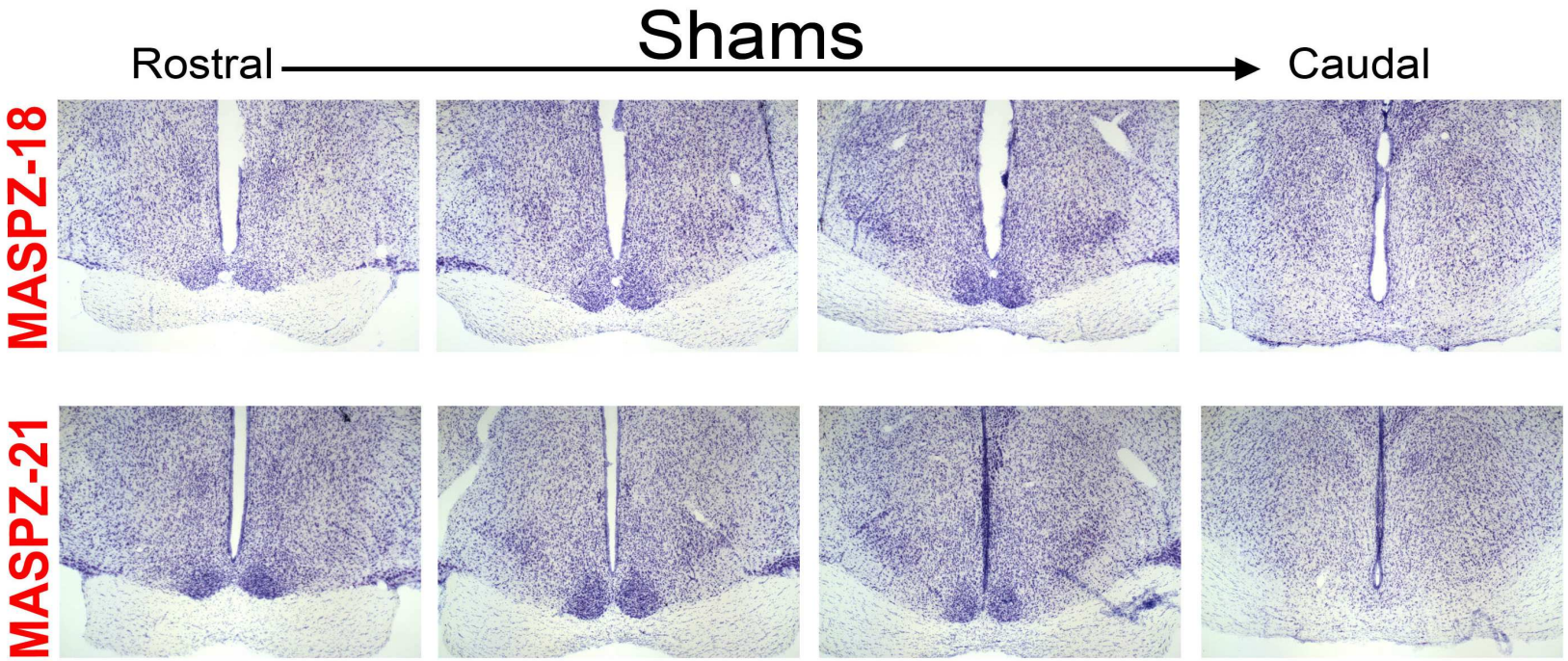


Figure 3.2. Photomicrographs of Nissl-stained tissue in 2 representative Miss animals and all unilaterally lesioned animals. Sections were selected to represent one section before the vSPZ, three spanning the rostral-caudal extent of the regions and one section after. Miss animals (MASPZ-13 & MASPZ-23) had no damage observed in the vSPZ or SCN, while unilaterally lesioned animals (MASPZ-05, MASPZ-08, & MASPZ-22) had one side of the vSPZ completely ablated. Areas damaged by the toxin are outlined in red.

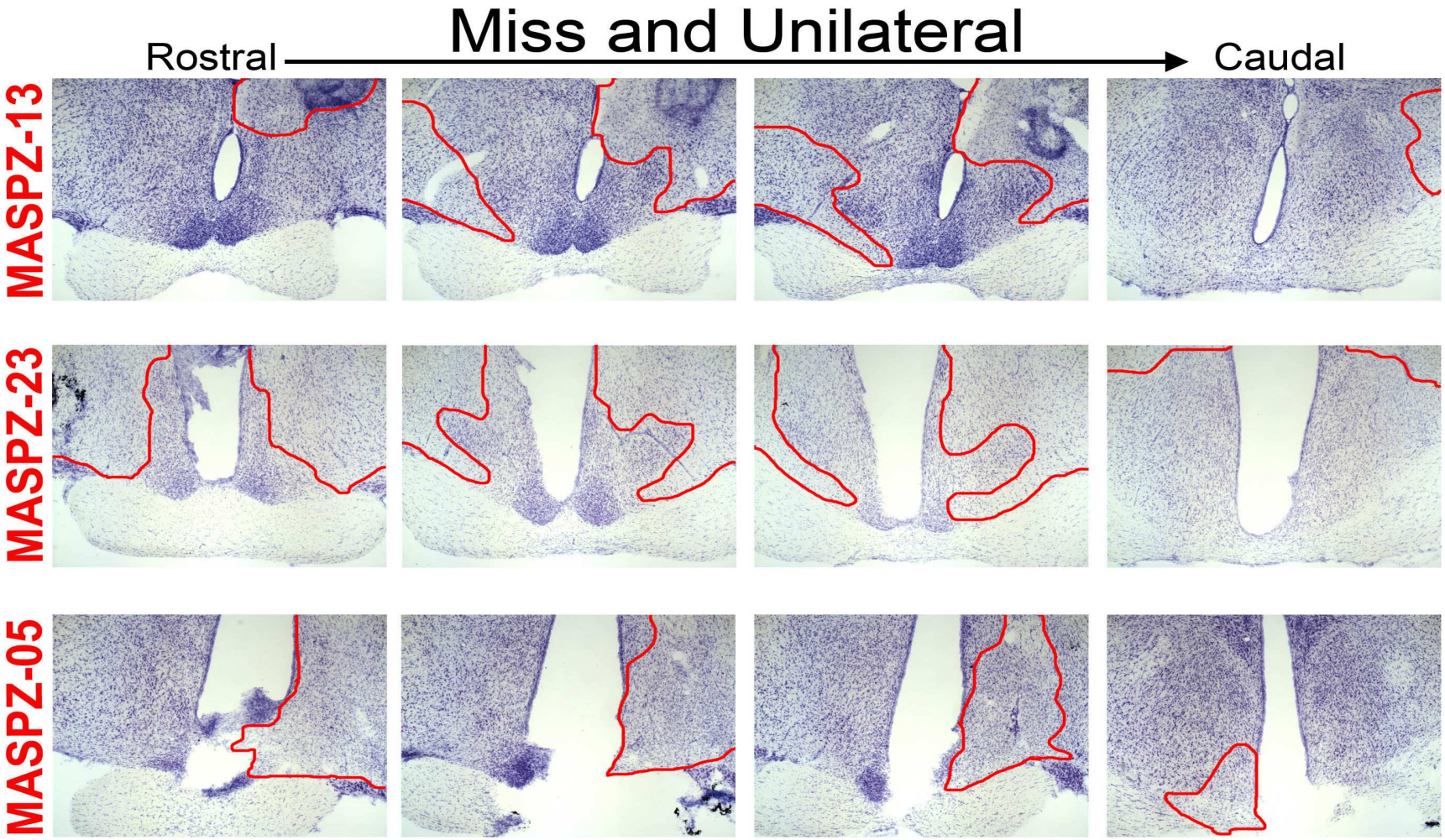


Figure 3.2. (cont'd)

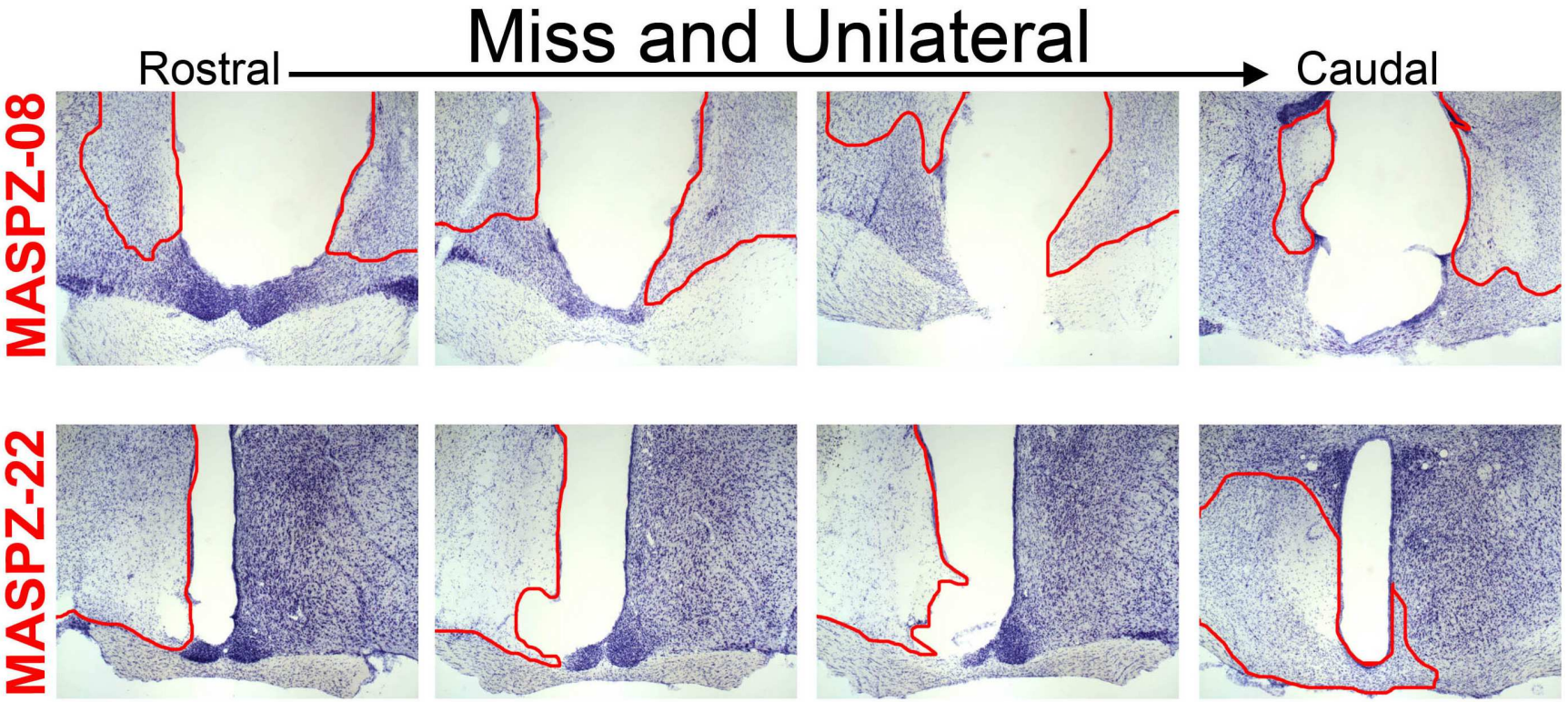
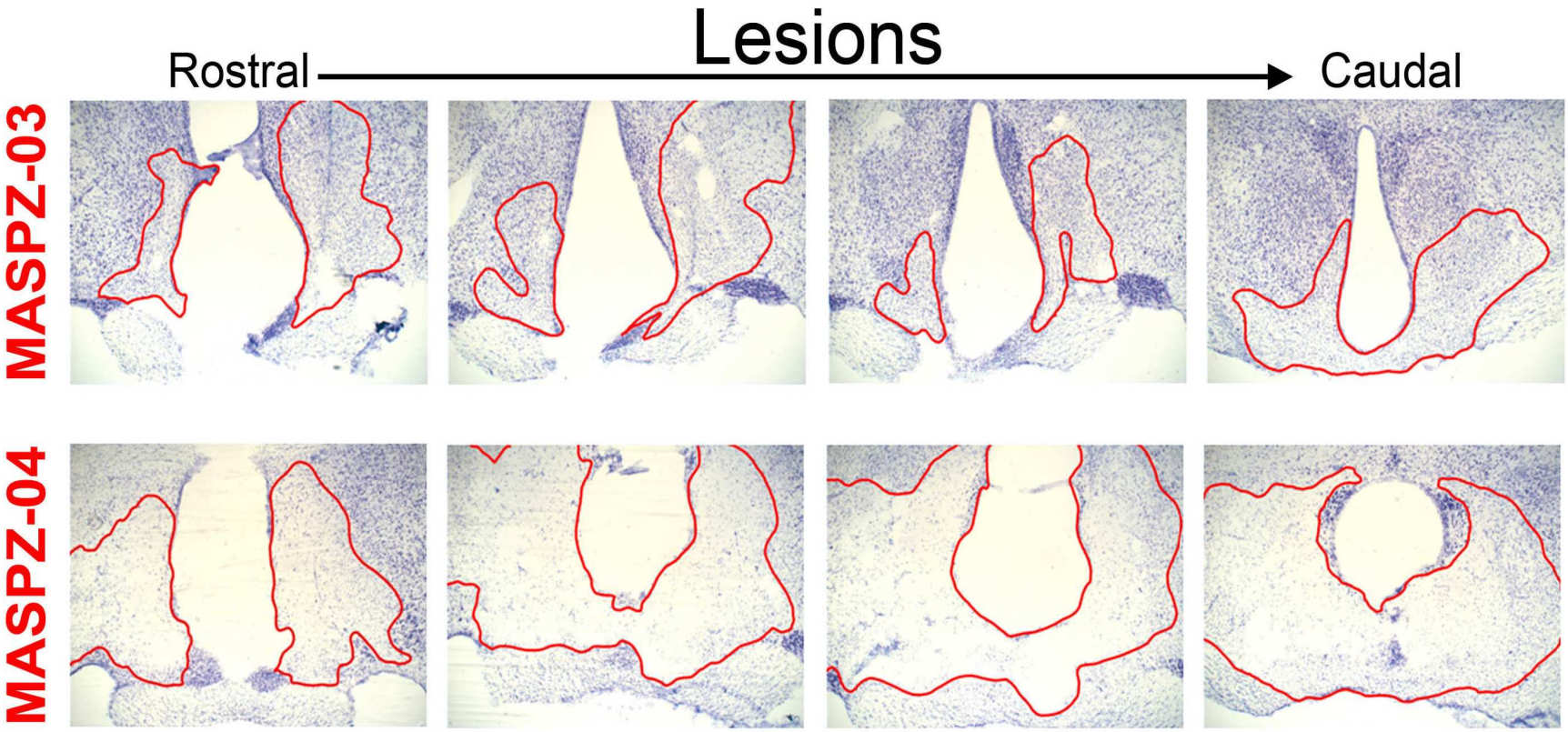


Figure 3.3. Photomicrographs of Nissl-stained tissue in all bilaterally lesioned animals. Sections were selected to represent one section before the vSPZ, three spanning the rostral-caudal extent of the regions and one section after. All lesioned animals had extensive damage observed in the vSPZ, 2 of these animals also had damage within the SCN (MASPZ-03 & MASPZ-04). Areas damaged by the toxin are outlined in red.



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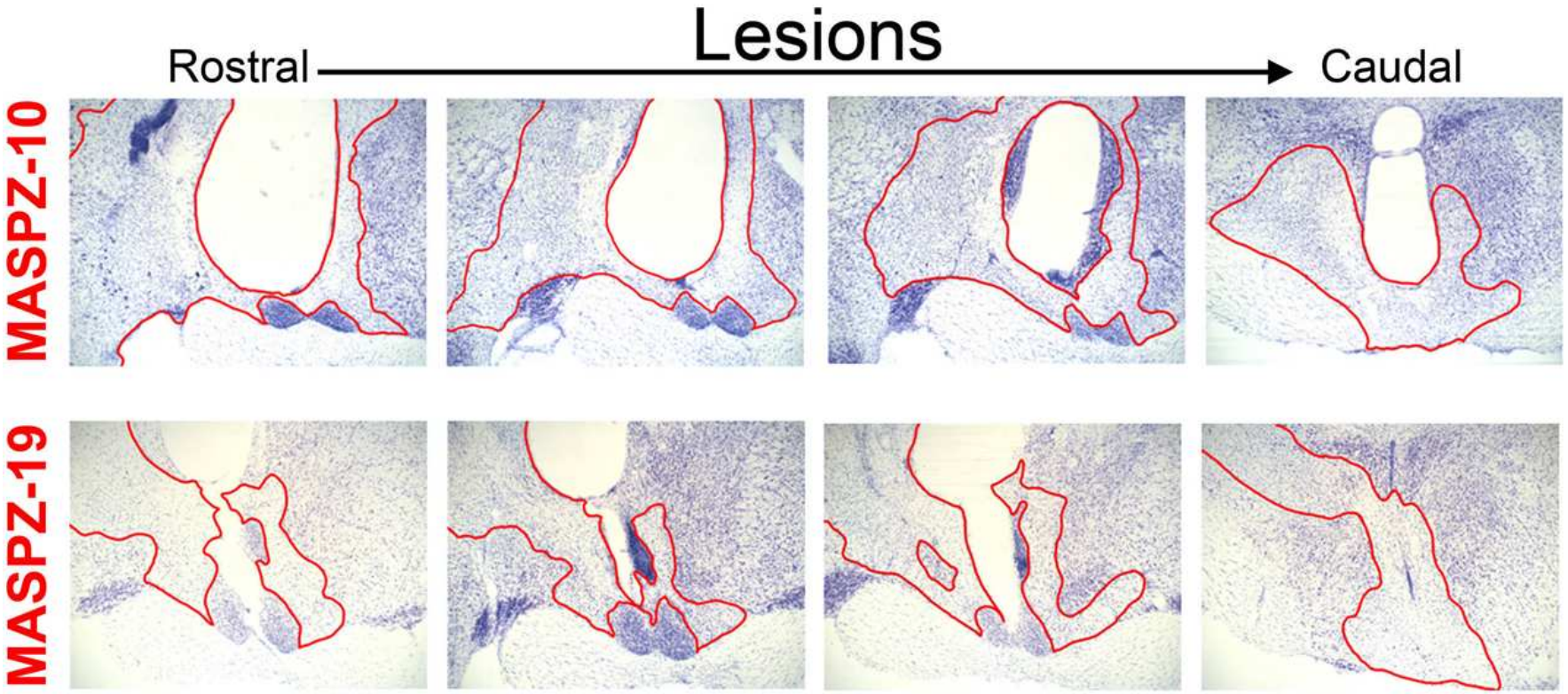
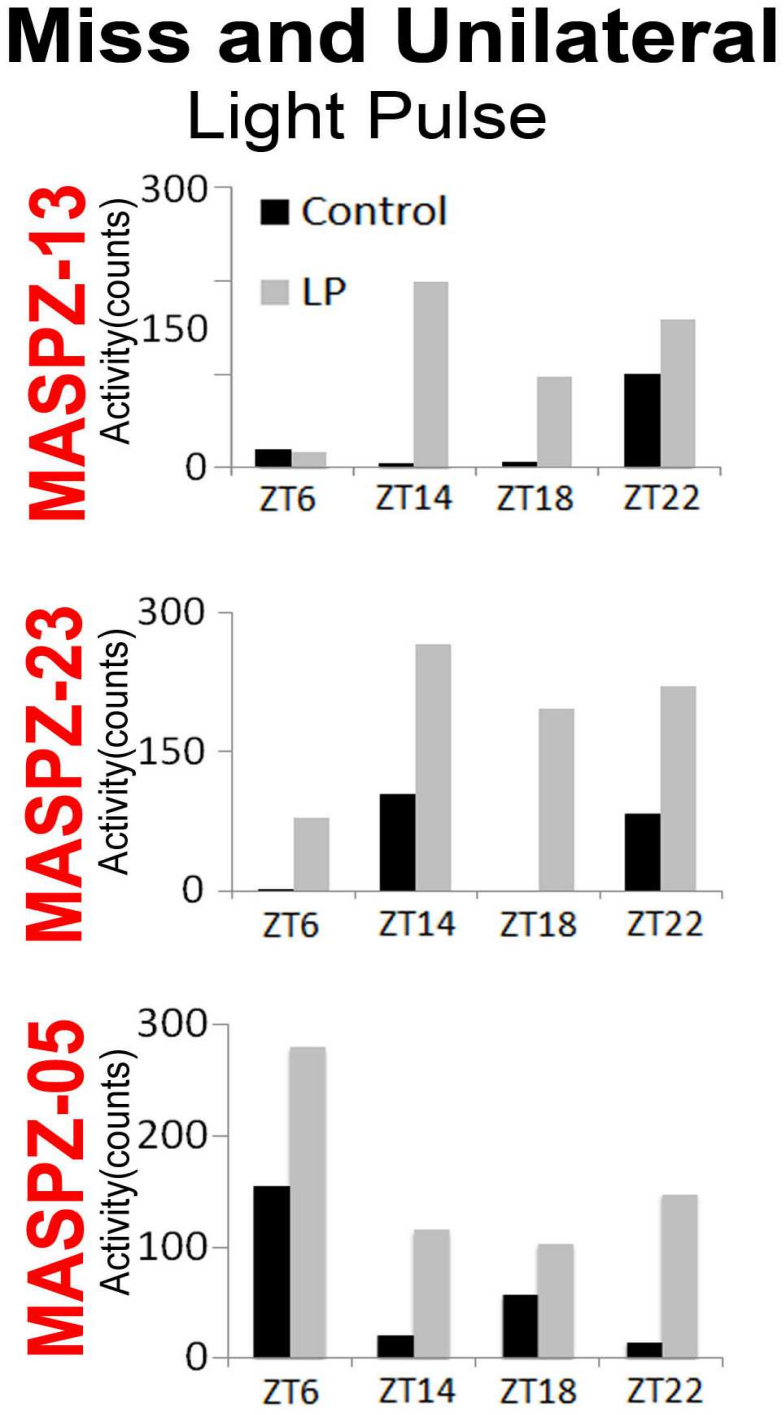


The analysis of the variation between groups for the ultradian cycle also revealed that it violated the assumption of sphericity. Only in the miss group was there a significant difference in activity between the lights on and lights off conditions ($t(6)=3.067$, $p=0.028$). Within this group all individuals exhibited higher activity levels during the light period and displayed 24 hr rhythms, and in one individual a 7 hr rhythm was evident in the chi-squared periodogram (Figure 3.7). Among unilateral (Figure 3.7) and sham groups (Figure 3.8) there was no difference in activity between dark and light periods. In the sham animals, 3 grass rats had little or no difference between activity in the light and dark periods (MASPZ-07, MASPZ-15, and MASPZ-18; Figure 3.8) while the other two were more active during the light (MASPZ-14 and MASPZ-21). Among bilaterally lesioned animals 3 of 4 individuals were more active during the light than dark periods (Figure 3.9). Only one grass rat in this group showed a significant 7 hr rhythm; this animal also showed the greatest masking response to the 1 hr light pulses (MASPZ-03). The other 3 animals with bilateral lesions had no 7 hr cycling, two had 24 hr rhythms and one was arrhythmic. Among animals in this group that had a significant 24 hr rhythm, activity was higher in the light than dark (MASPZ-03, MASPZ-10 and MASPZ-19), whereas among the arrhythmic animal the activity was higher during the dark than the light (MASPZ-04; Figure 3.9).

Circadian Rhythms

Analysis of the variance in τ in constant darkness and in constant light again showed that it violated the assumption of sphericity. Paired-sample t-tests of the

Figure 3.4. Patterns of behavioral response of activity to light exposure in Miss and Unilateral animals. On the x-axis, 4 different time points, ZT6, ZT14, ZT18 and ZT22, were examined. The black bars represent acivity during 1 hr of a control day while grey bars are the same hour during the light exposure. All animals respond to light at most time points (16/20), however, two animals had a time-point where there was no response (MASPZ-13 & MASPZ-22) and one animal had two time points were activity was decreased (MASPZ-08).



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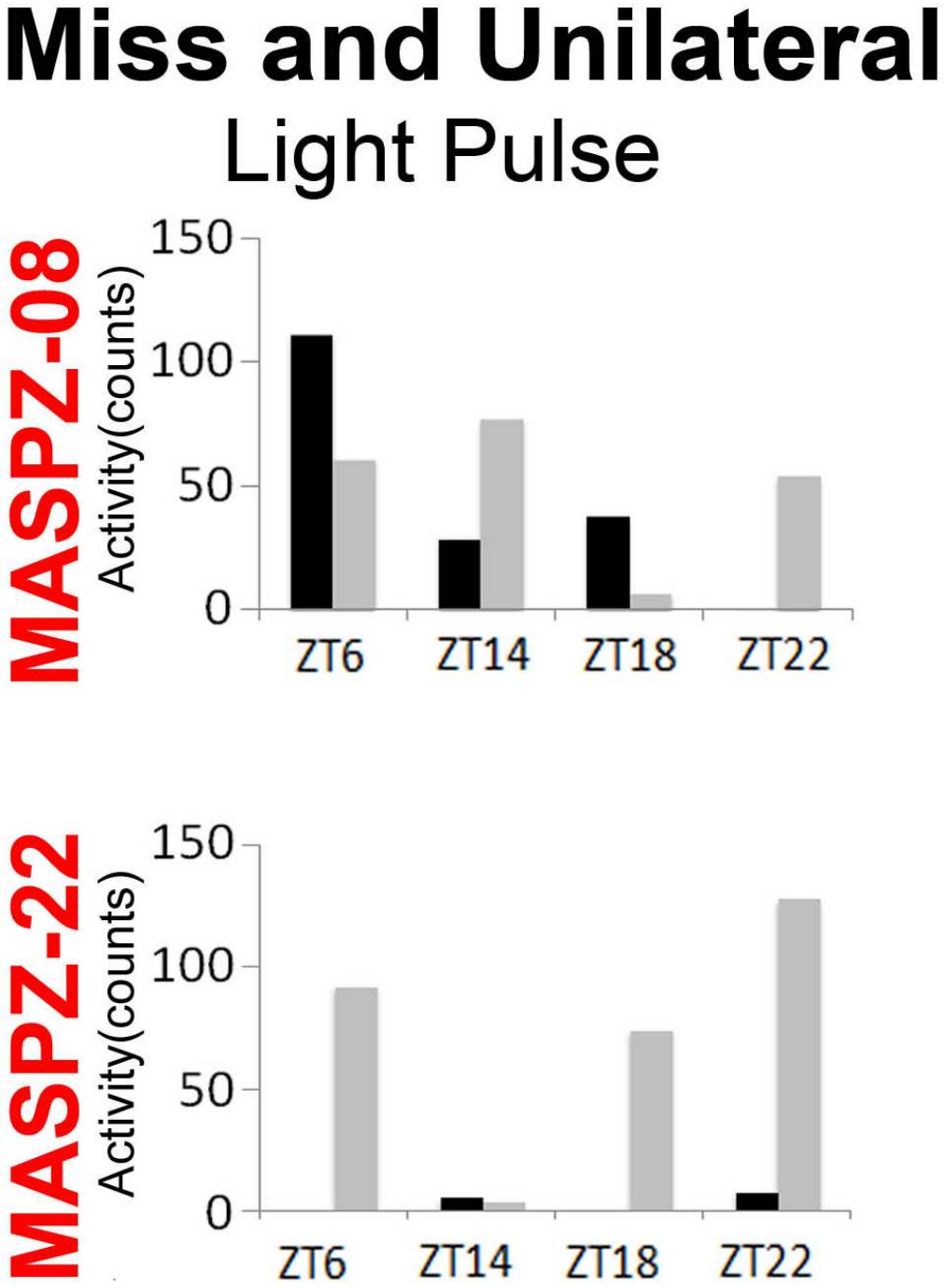
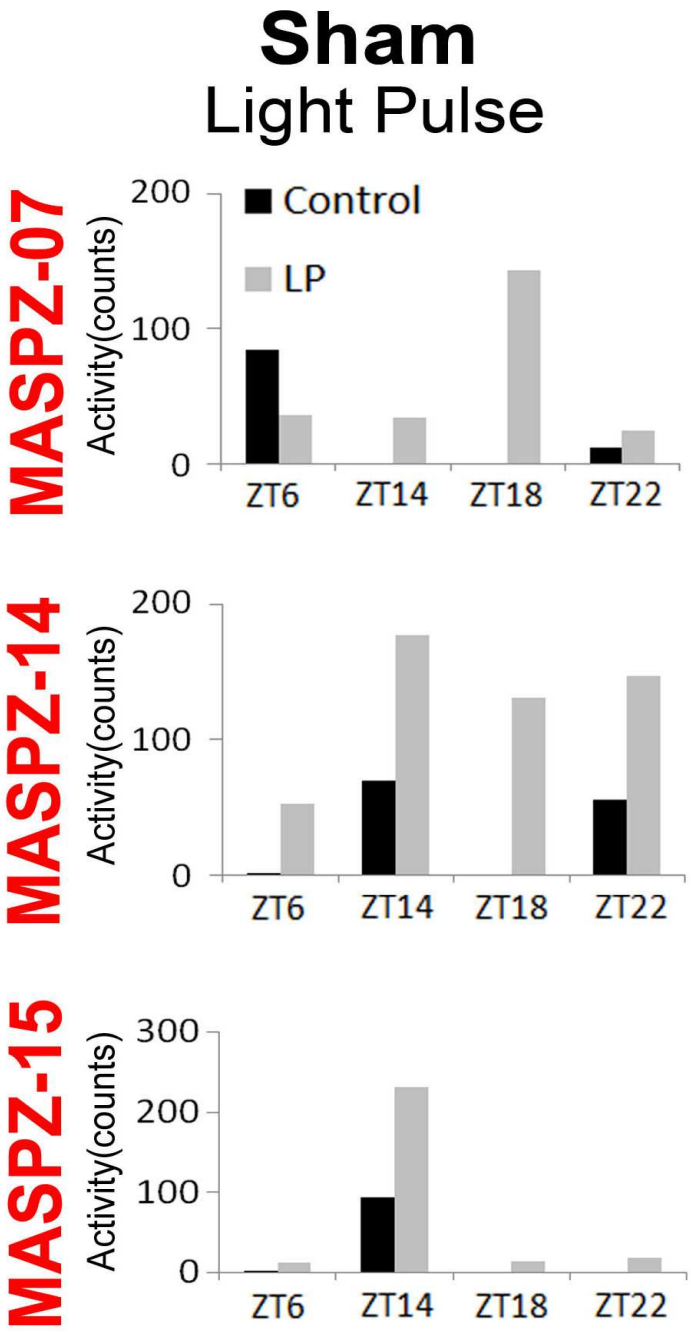


Figure 3.5. Patterns of behavioral response of activity to light exposure in SHAM animals. On the x-axis, 4 different time points, ZT6, ZT14, ZT18 and ZT22, were examined. The black bars represent acivity during 1 hr of a control day while grey bars are the same hour during the light exposure. Two animals had strong positive masking behavior, with an increase for all time points (MASPZ-14 & MASPZ-21). One animal did not display a constant masking response, with both increases and decreases in activity (MASPZ-07). The two final animals had very low activity level readings during this portion of the experiment making it hard to interpret their masking behavior (MASPZ-15 & MASPZ-18).



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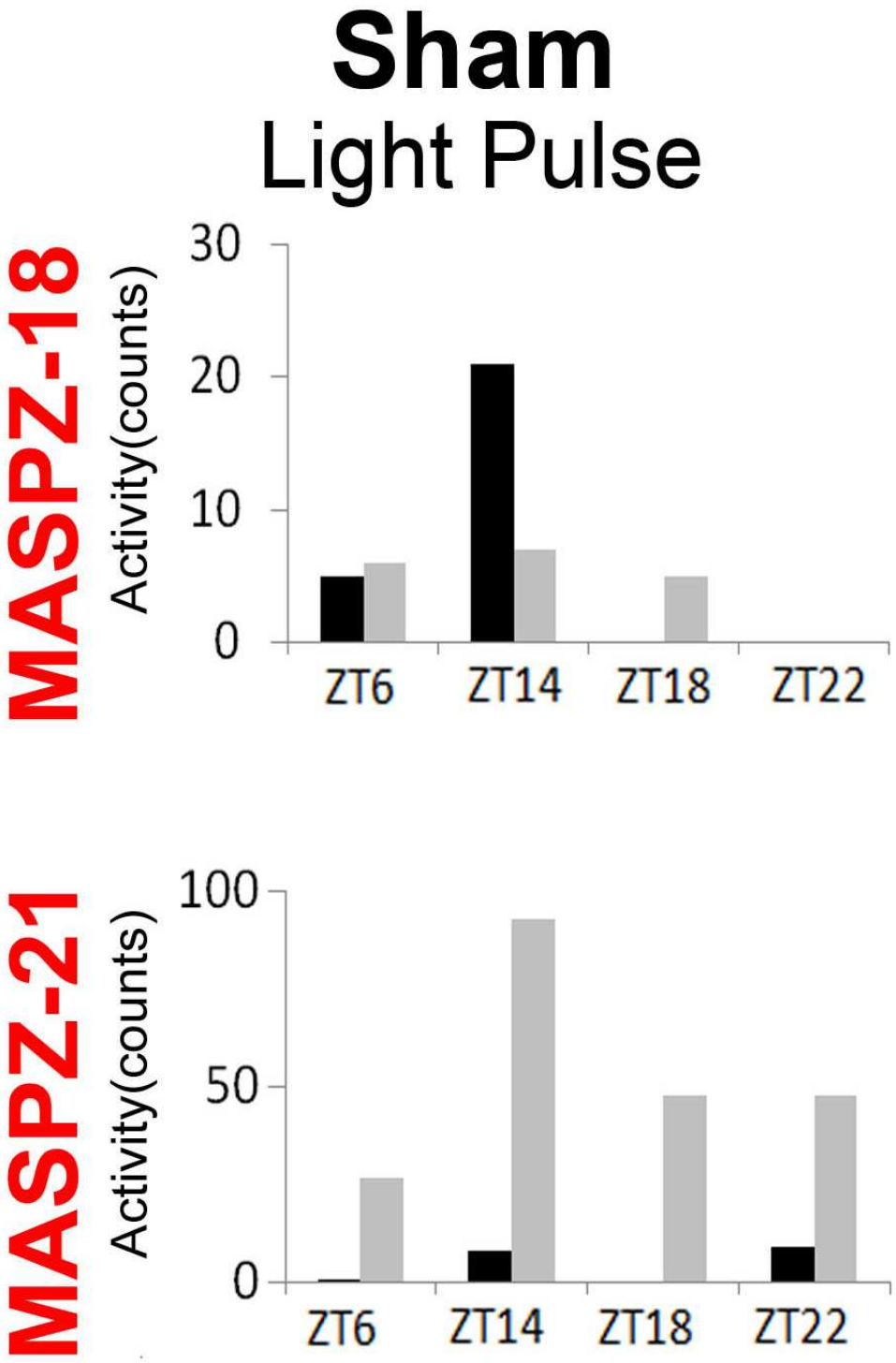
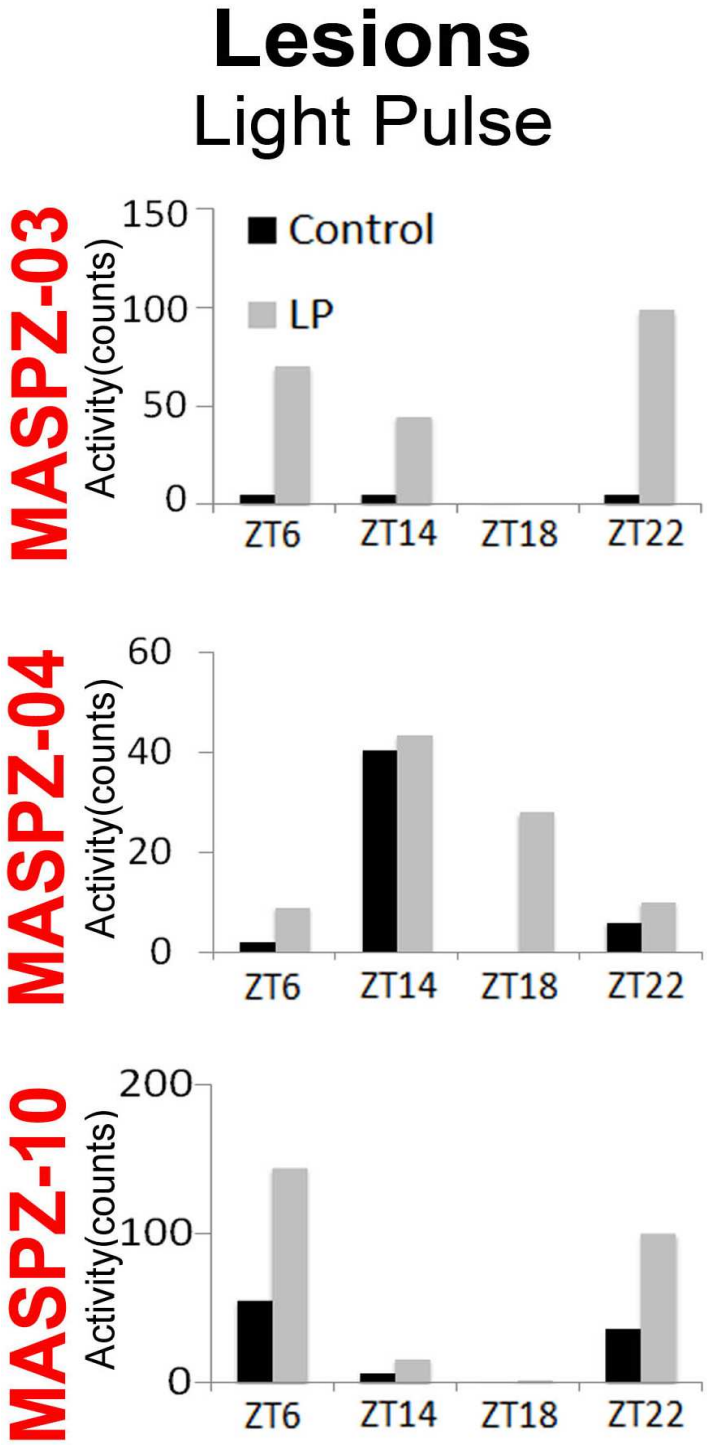


Figure 3.6. Patterns of behavioral response of activity to light exposure in Miss and Unilateral animals. On the x-axis, 4 different time points, ZT6, ZT14, ZT18 and ZT22, were examined. The black bars represent acivity during 1 hr of a control day while grey bars are the same hour during the light exposure. All animals respond to light at most time points (12/16), however, two animals had a time point where there was no response (MASPZ-03 & MASPZ-10) and one animal had two time points were activity was decreased (MASPZ-19).



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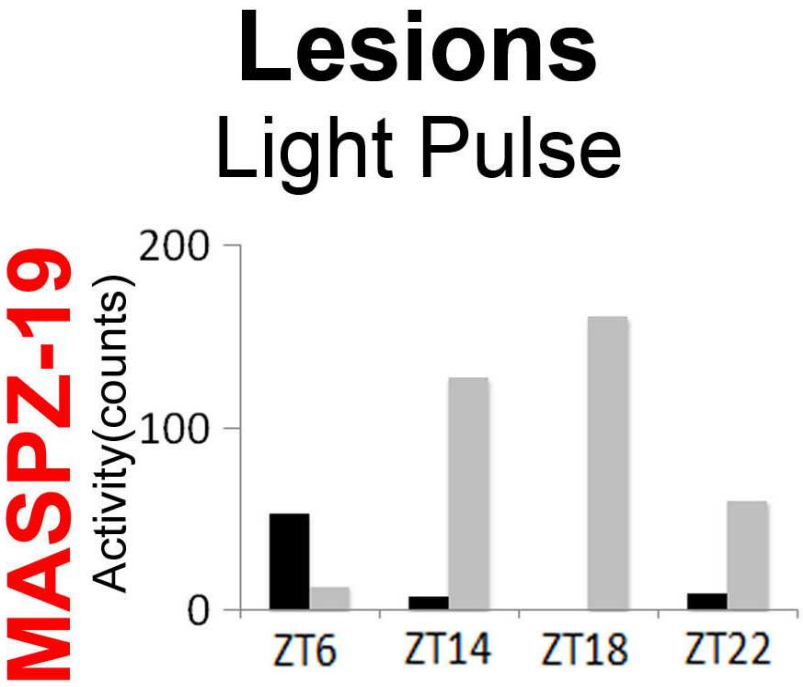


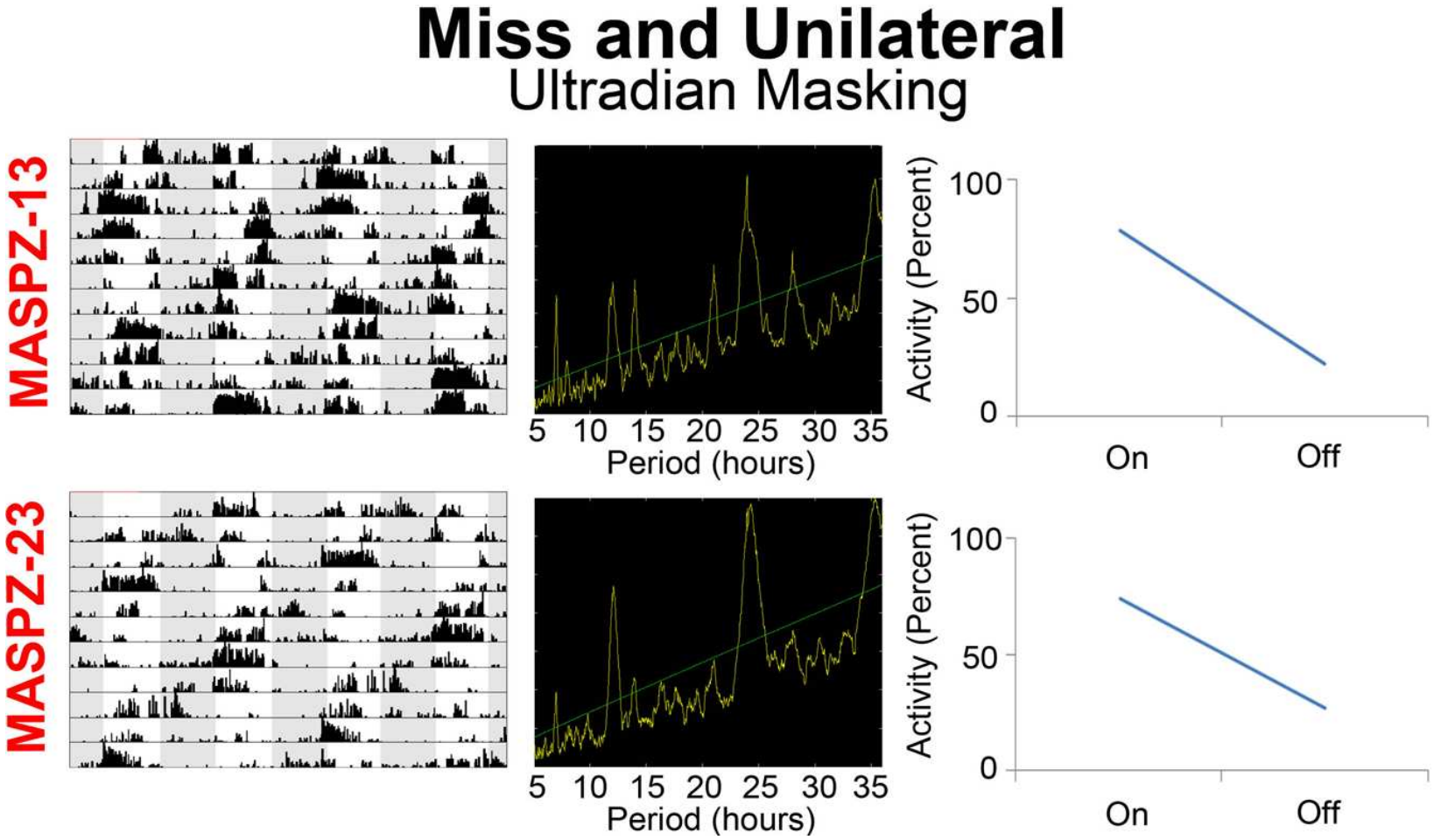
remaining groups showed a significantly longer τ in LL when compared to DD for the miss group ($t(4)=4.453$, $p=0.011$) and a trend toward significance for the sham group ($t(4)=2.482$, $p=0.068$). The Chi-squared analysis showed the presence of circadian rhythms in all individuals in sham (Figure 3.10), unilateral lesion, and miss groups (Figure 3.11) but not in all bilaterally lesioned animals (Figure 3.12). Three of the 4 animals in this group had rhythms in LD conditions, however, this decreased to 2 in DD and 1 in LL. Therefore, there was greater variance in the bilateral lesion group, which led to the violation of the assumption of sphericity. This was also the case for the analysis of the alpha/rho ratio in this group. Separate one-way ANOVAs of the data from the miss ($F[2, 16] = 63.382$, $p < 0.001$) and sham ($F[2, 8] = 9.056$, $p = 0.012$) animals both showed a significantly higher alpha/rho ratio in LL compared to DD and LD.

DISCUSSION

In this experiment we lesioned the vSPZ, a structure found deep the hypothalamus, in diurnal grass rats, and assessed masking behavior as well as circadian rhythms in these animals. Previous attempts to ablate the vSPZ in grass rats yielded no complete lesions but demonstrated a relationship between lesion size and rhythm dysfunction. We were able to successfully destroy the vSPZ but these lesions had no effect on the masking response to light.

Masking

Figure 3.7. 3.5:3.5 LD Ultradian masking protocol in Miss and Unilateral animals. The far left panel shows the 28hr actograms of animals during the ultradian cycle, grey bars indicate the dark phases of the cycle. The middle panel shows the Chi-Squared periodogram of animals during the last 7 days of the protocol. All animals have a significant 24 hr rhythm and only one animal has a significant 7 hr cycle (MASPZ-13). On the far right panel, total activity during the light and dark period were summed and turned into a percentage of activity. All animals have the expected higher percentage of activity during the light phases with the exception of MASPZ-08, who had more activity during the dark phases.



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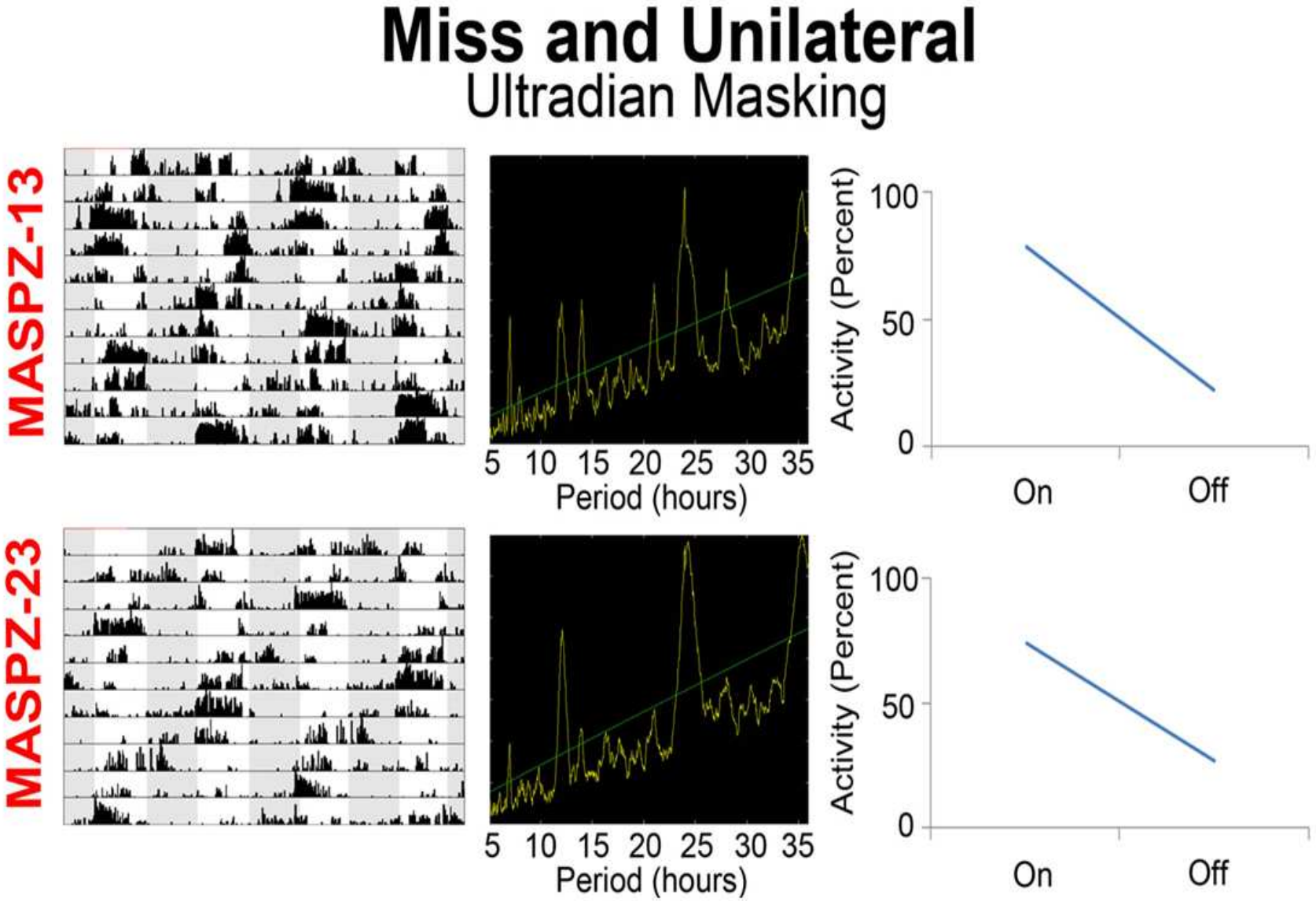

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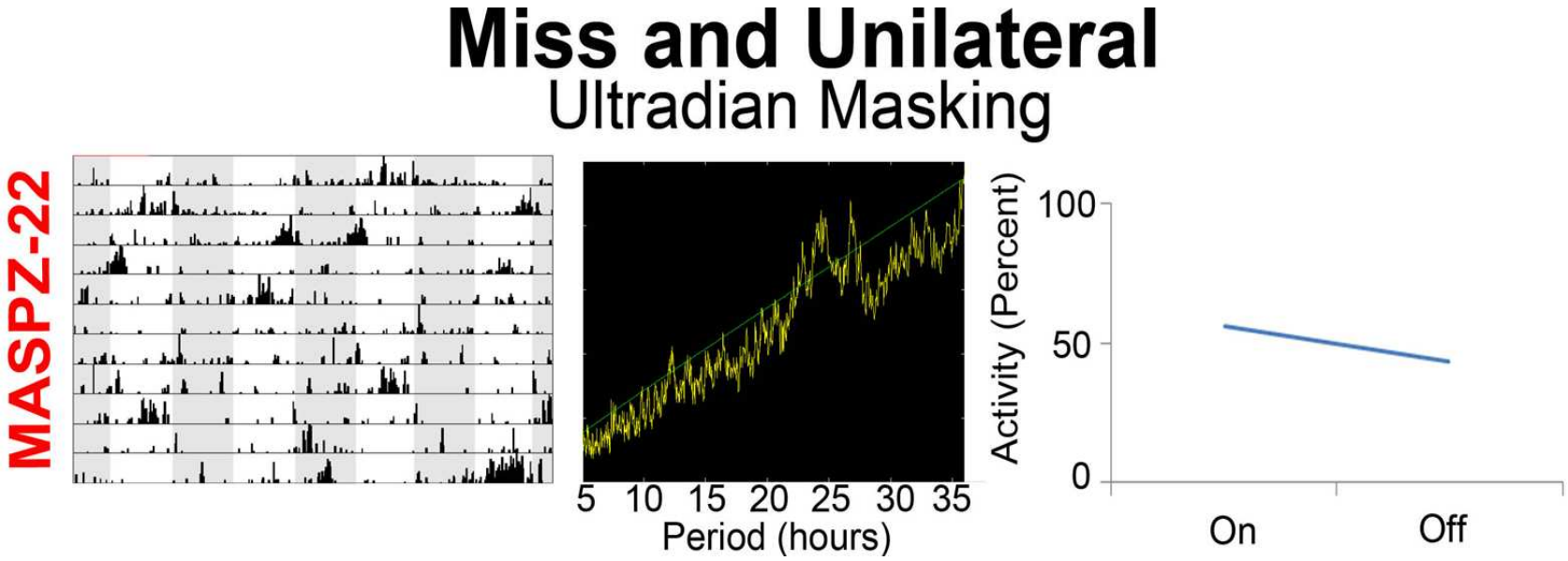
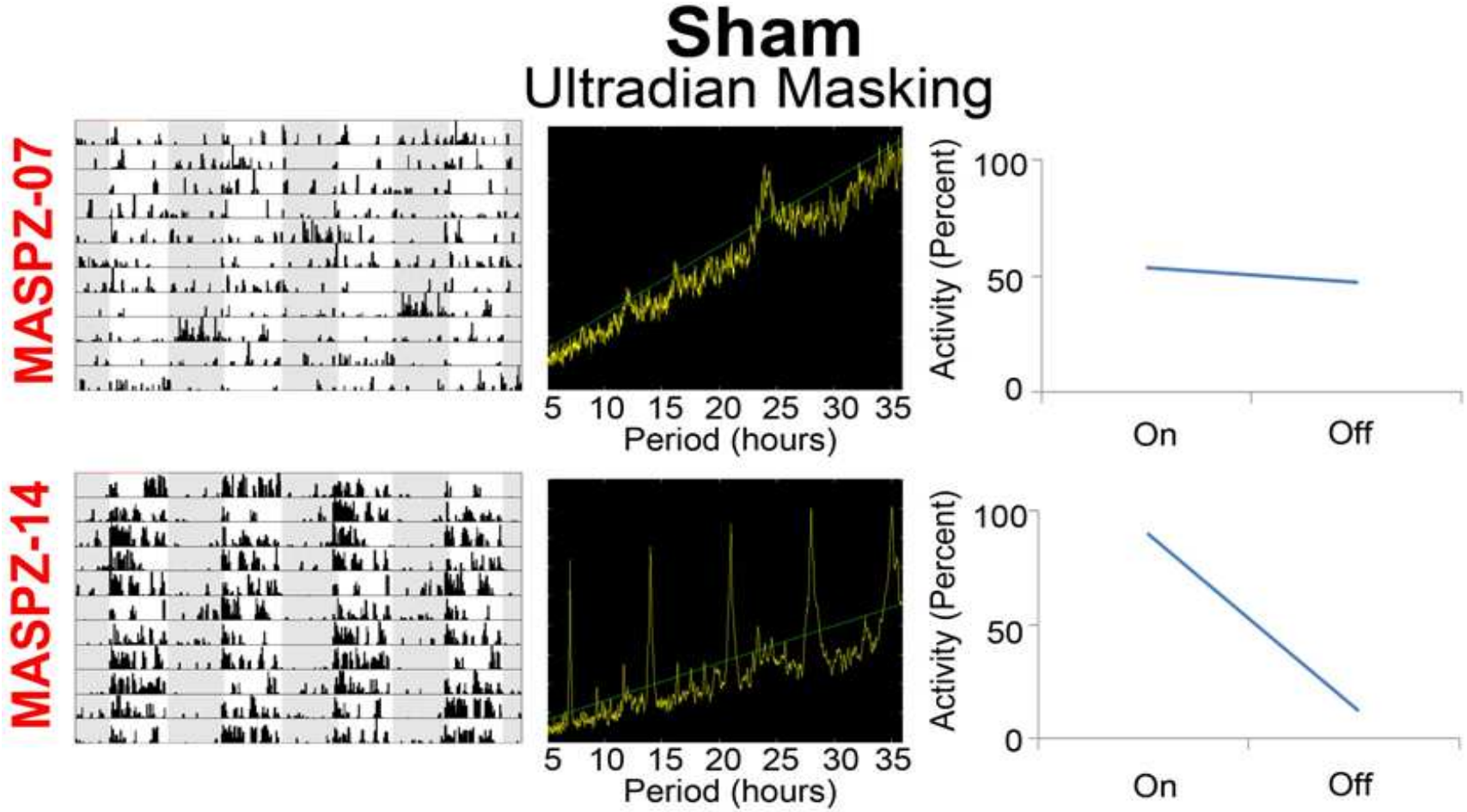


Figure 3.8. 3.5:3.5 LD Ultradian masking protocol in SHAM animals. The far left panel shows the 28hr actograms of animals during the ultradian cycle, grey bars indicate the dark phases of the cycle. The middle panel shows the Chi-Squared periodogram of animals during the last 7 days of the protocol. All animals have a significant 24 hr rhythm and only one animal has a significant 7 hr cycle (MASPZ-14). On the far right panel, total activity during the light and dark period were summed and turned into a percentage of activity. Only 2 animals (MASPZ-14 & MASPZ-21) have the expected higher percentage of activity during the light phases, the other 3 animals (MASPZ-7, MASPZ-15 & MASPZ-18) did not respond with equal amounts of activity during the light and dark phases.



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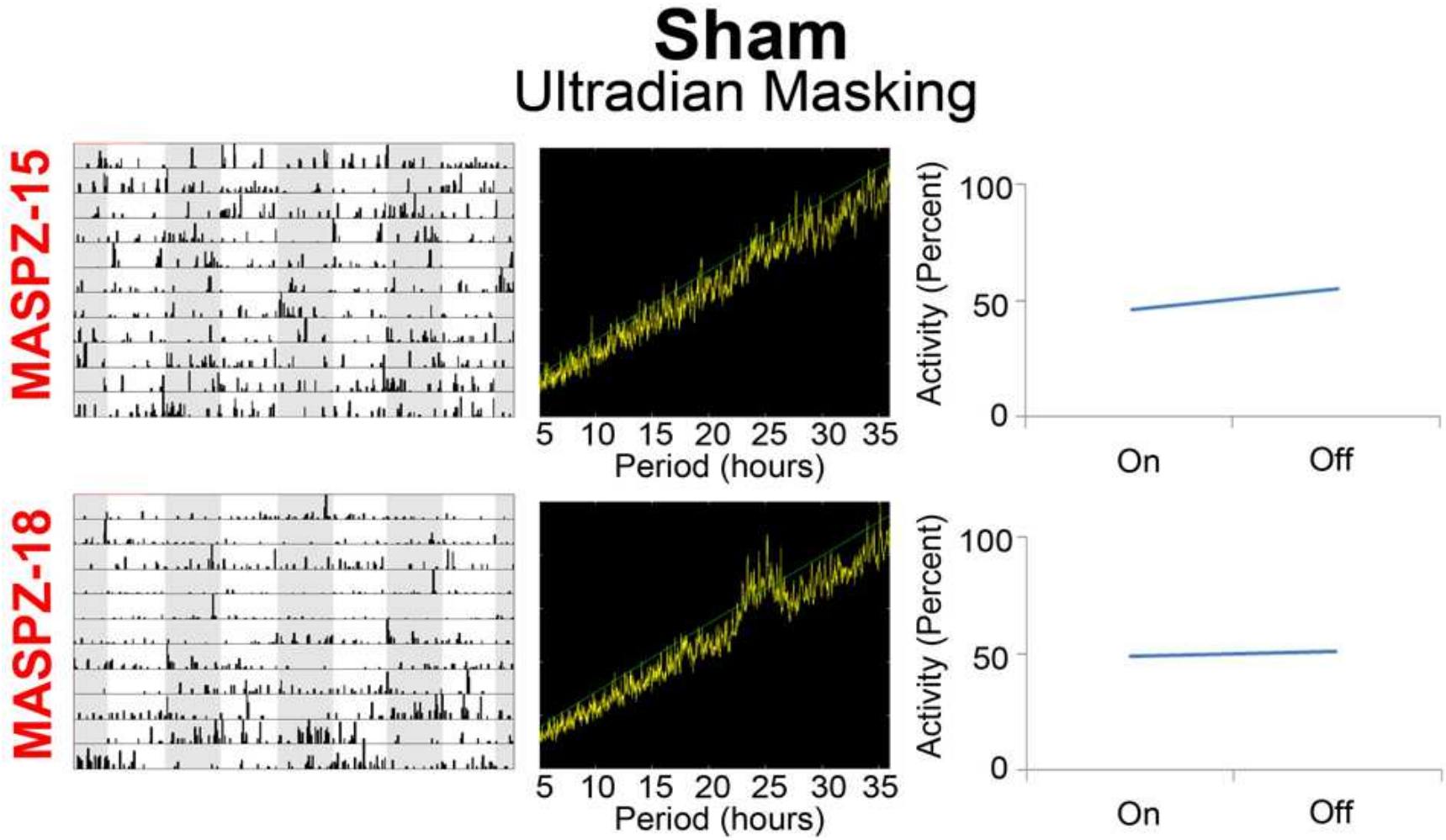

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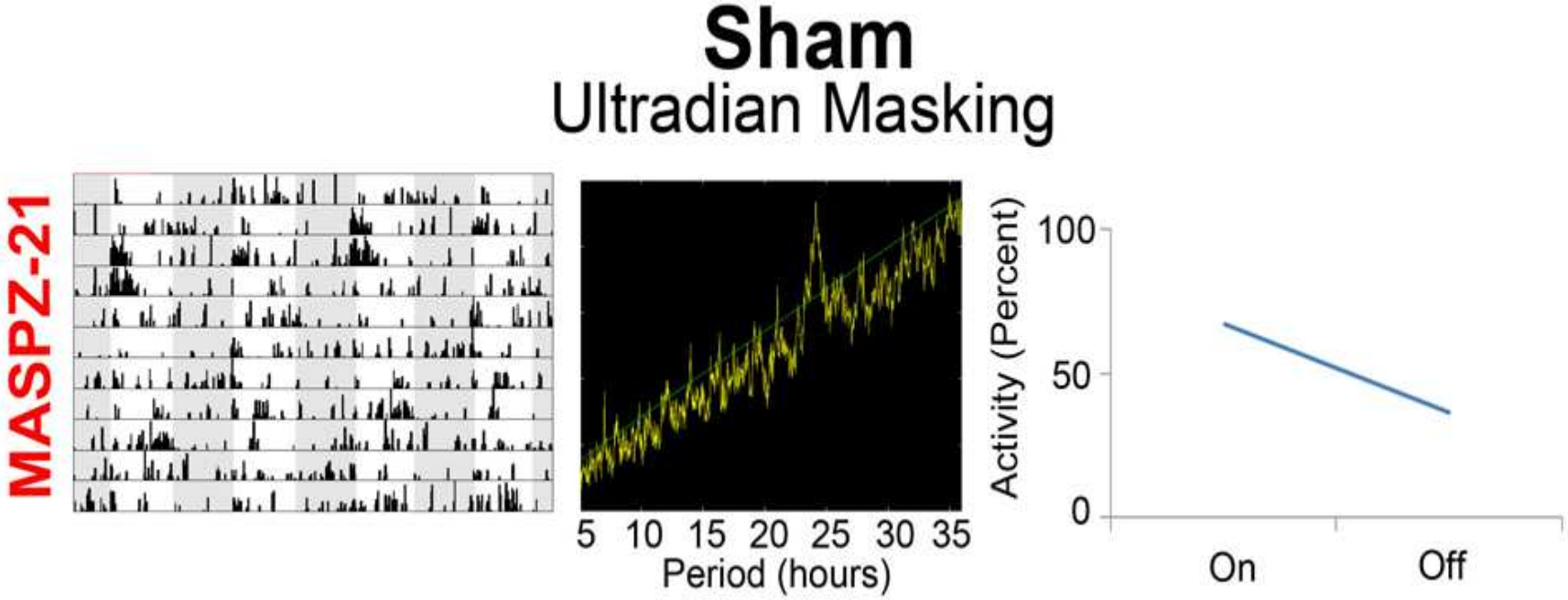
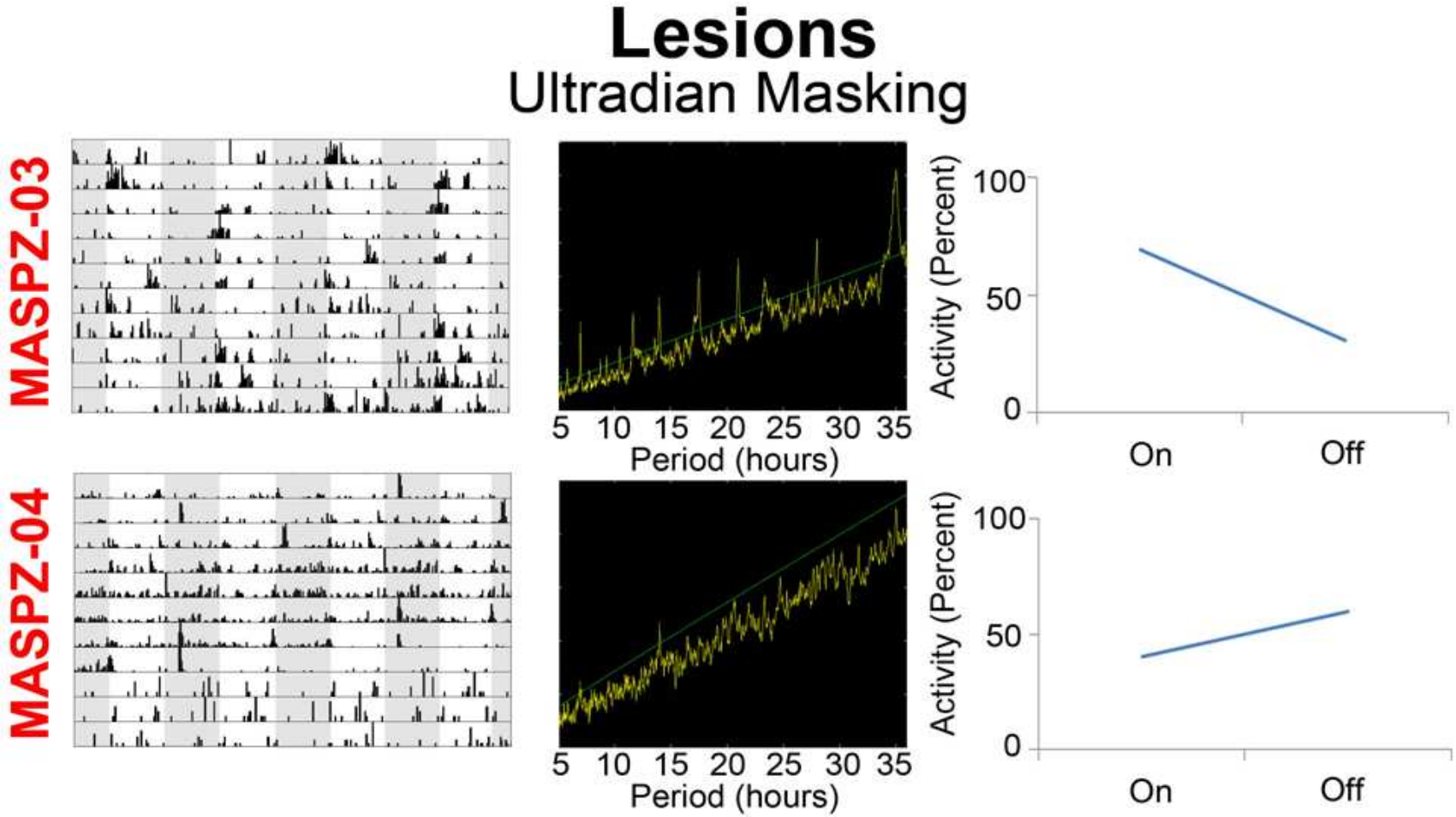


Figure 3.9. 3.5:3.5 LD Ultradian masking protocol in SHAM animals. The far left panel shows the 28hr actograms of animals during the ultradian cycle, grey bars indicate the dark phases of the cycle. The middle panel shows the Chi-Squared periodogram of animals during the last 7 days of the protocol. Three animals have a significant 24 hr rhythm and one animal is arrhythmic (MASPZ-04). Only one animal had a significant 7 hr cycle (MASPZ-03). On the far right panel, total activity during the light and dark period were summed and turned into a percentage of activity. Three animals (MASPZ-03, MASPZ-10 & MASPZ-19) had the expected higher percentage of activity during the light phases, the other animals (MASPZ-04) did not respond and had higher activity during the dark phases.



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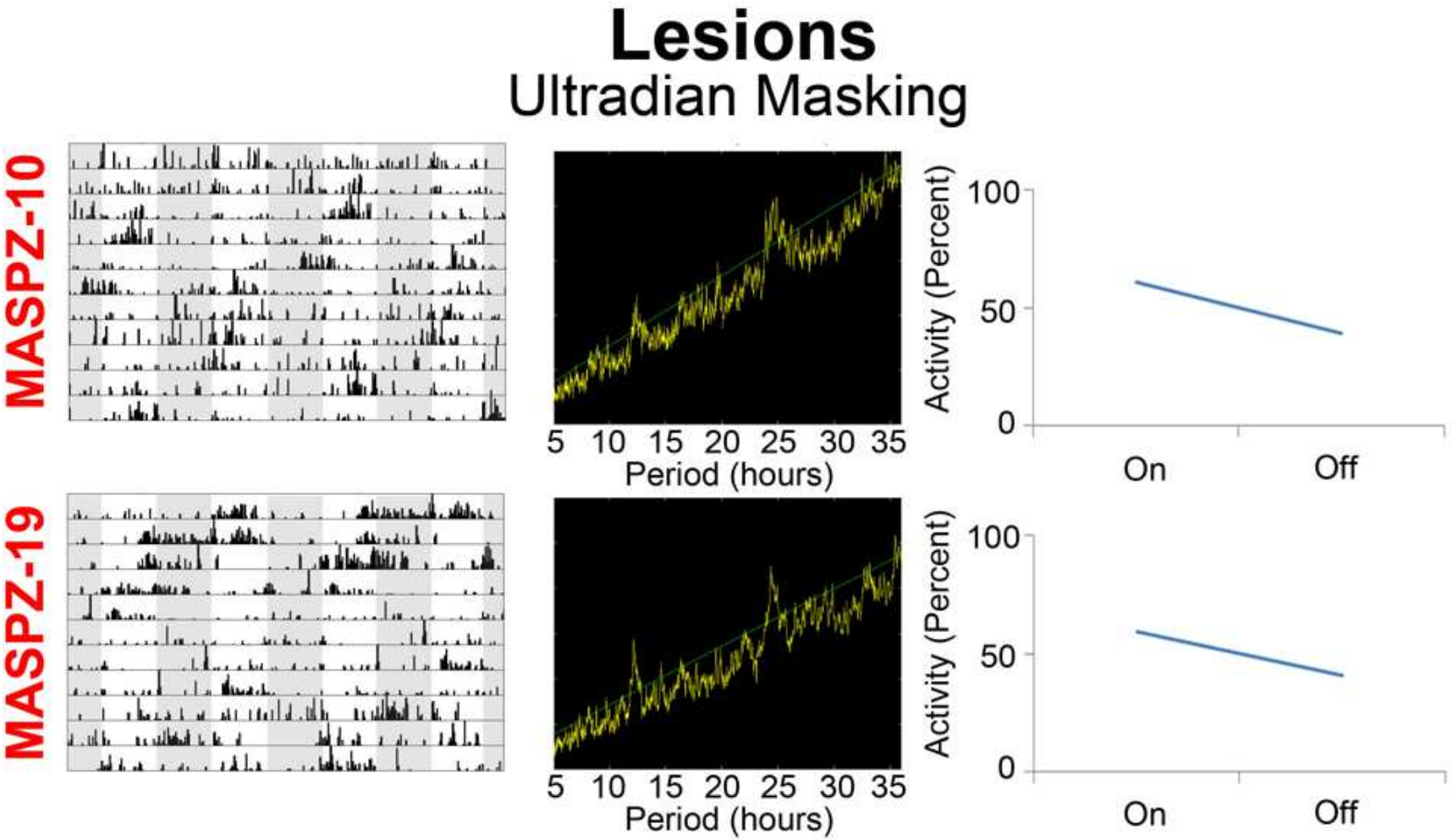
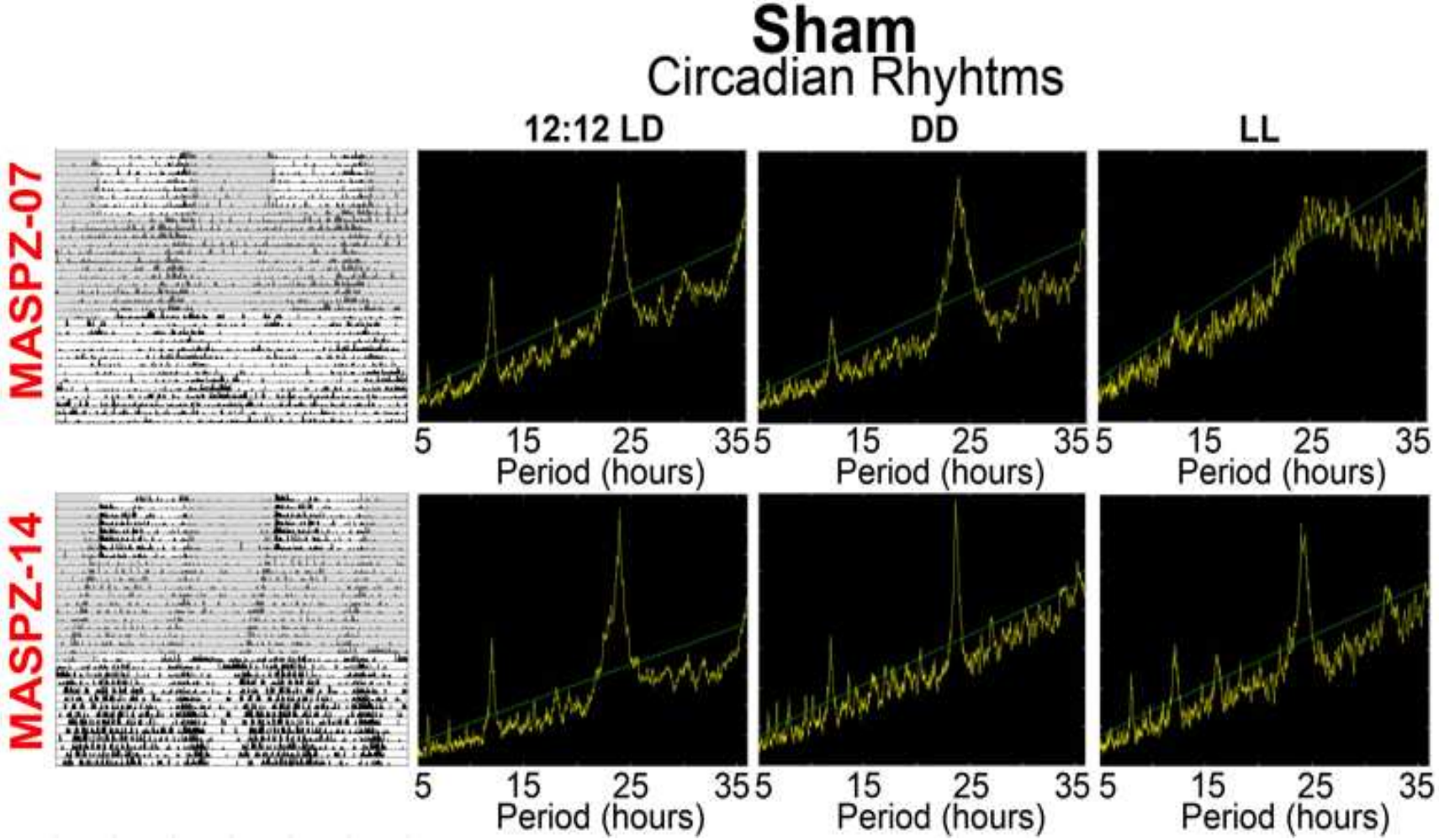


Results from both approaches that we took for the assessment of masking revealed that some animals in which the vSPV was completely destroyed were able to exhibit masking. First, in animals with bilateral lesions of the vSPZ there was a near significant increase in activity in response to a 1 hr pulse of light (p = 0.056). When we consider the 4 light pulses in all of the 4 animals with complete vSPZ lesions, we find that in only 1 of 16 cases was activity higher during the control period than during the light pulse, whereas in 13 of the 16 cases the reverse was true; on two occasions there was no difference. Secondly, a significant 7 hr rhythm in the ultradian LD cycle was observed in one individual (MASPZ-03; Figure 3.9) that sustained complete bilateral damage of the vSPZ; in this animal more activity occurred during the light than dark phase of the cycle. Furthermore, 3 of these 4 animals showed more activity in the light than dark phase of the ultradian cycle. Together, these data have established that the vSPZ is not necessary for masking.

There are no published reports of effects of vSPZ lesions on masking. However, it is impossible to destroy the SCN without damaging some of the vSPZ, and there are several studies that have examined effects of SCN lesions on masking. In one report of a study in which SCN lesions impaired masking in hamsters, in the context of a 7 h ultradian LD cycle, the authors noted that the effect could have been due to destruction of retinal fibers projecting to the vSPZ (Li et al., 2005). Additionally, in circadian rhythms studies in which the vSPZ was eliminated in rats (Abrahamson & Moore, 2006; Cipolla-Neto et al., 1995; Lu et al., 2001; Moore & Danchenko, 2002) animals continued to have nighttime activity in LD conditions after ablation of the region. Demonstrating that for nocturnal animals, which have a disrupted expression of activity rhythms in constant

Figure 3.10. Circadian rhythms analysis in varying lighting conditions in SHAM. On the Far left, actograms of all animals across three different lighting conditions, 12:12 LD, constant light, and constant darkness. Chi-squared analysis was conducted to detect the presence of significant rhythms were run for all lighting conditions. All animals had rhythms at the three lighting conditions except MASPZ-15 in LL.



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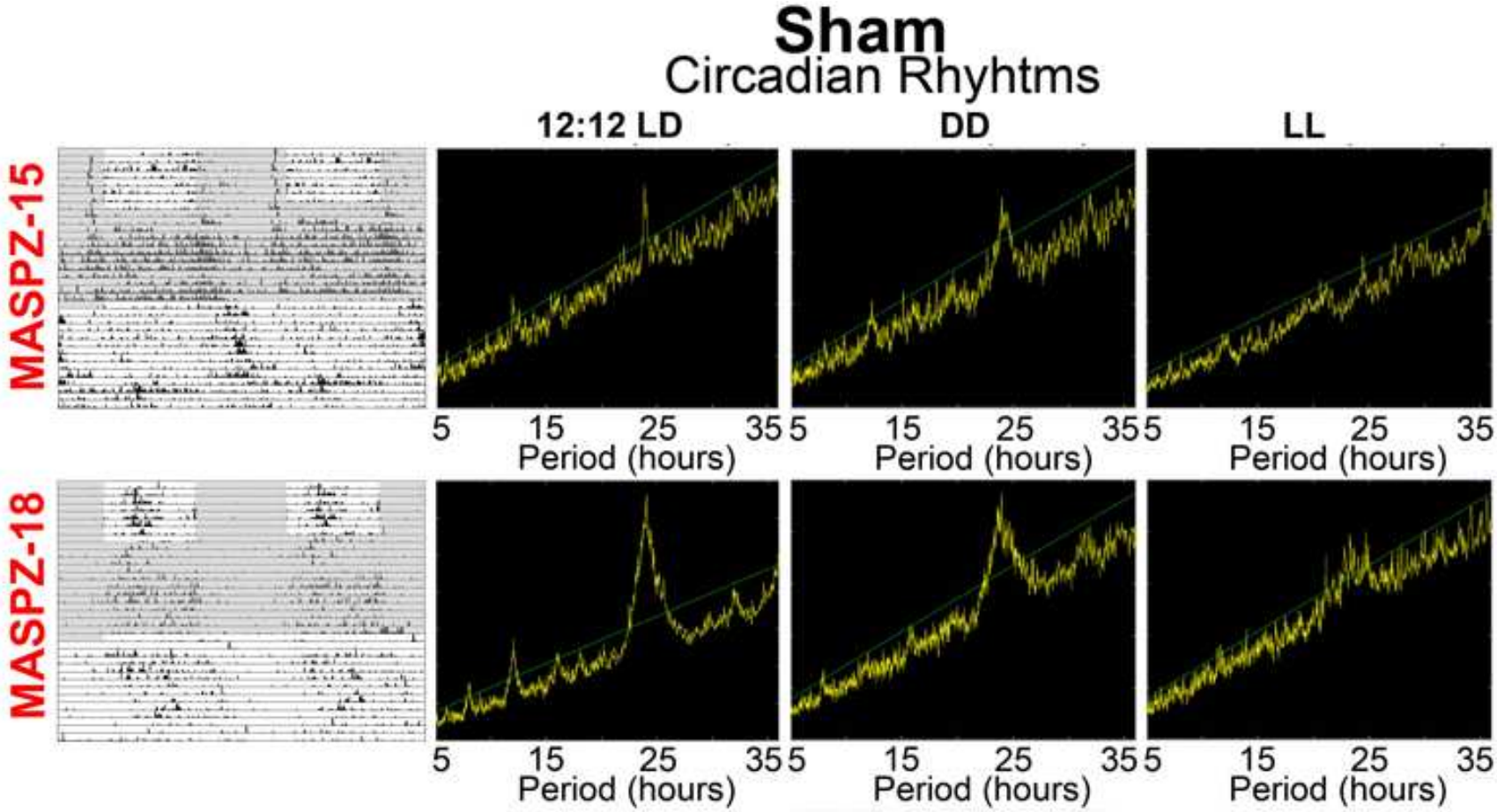

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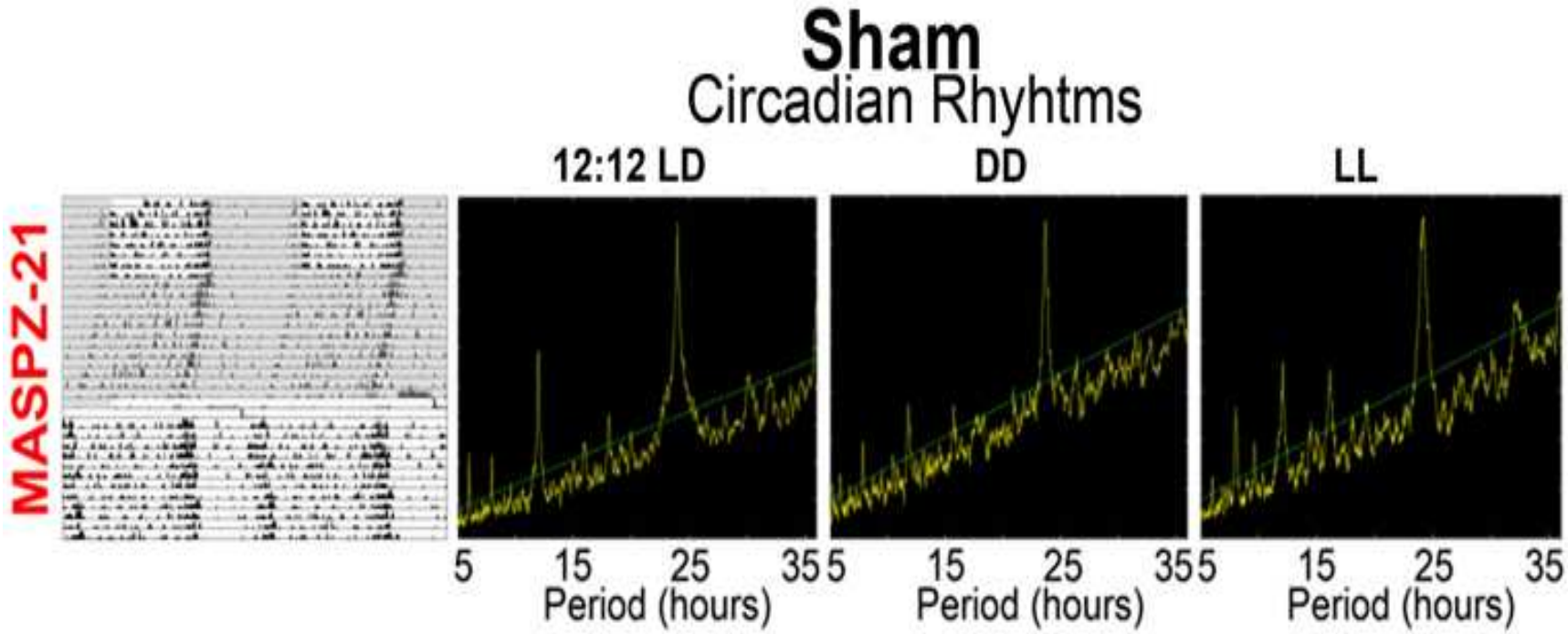


Figure 3.11. Circadian rhythms analysis in varying lighting conditions in Miss and Unilateral groups. On the Far left, actograms of all animals across three different lighting conditions, 12:12 LD, constant light, and constant darkness. Chi-squared analysis was conducted to detect the presence of significant rhythms were run for all lighting conditions. All animals had significant rhythms at the three lighting conditions.

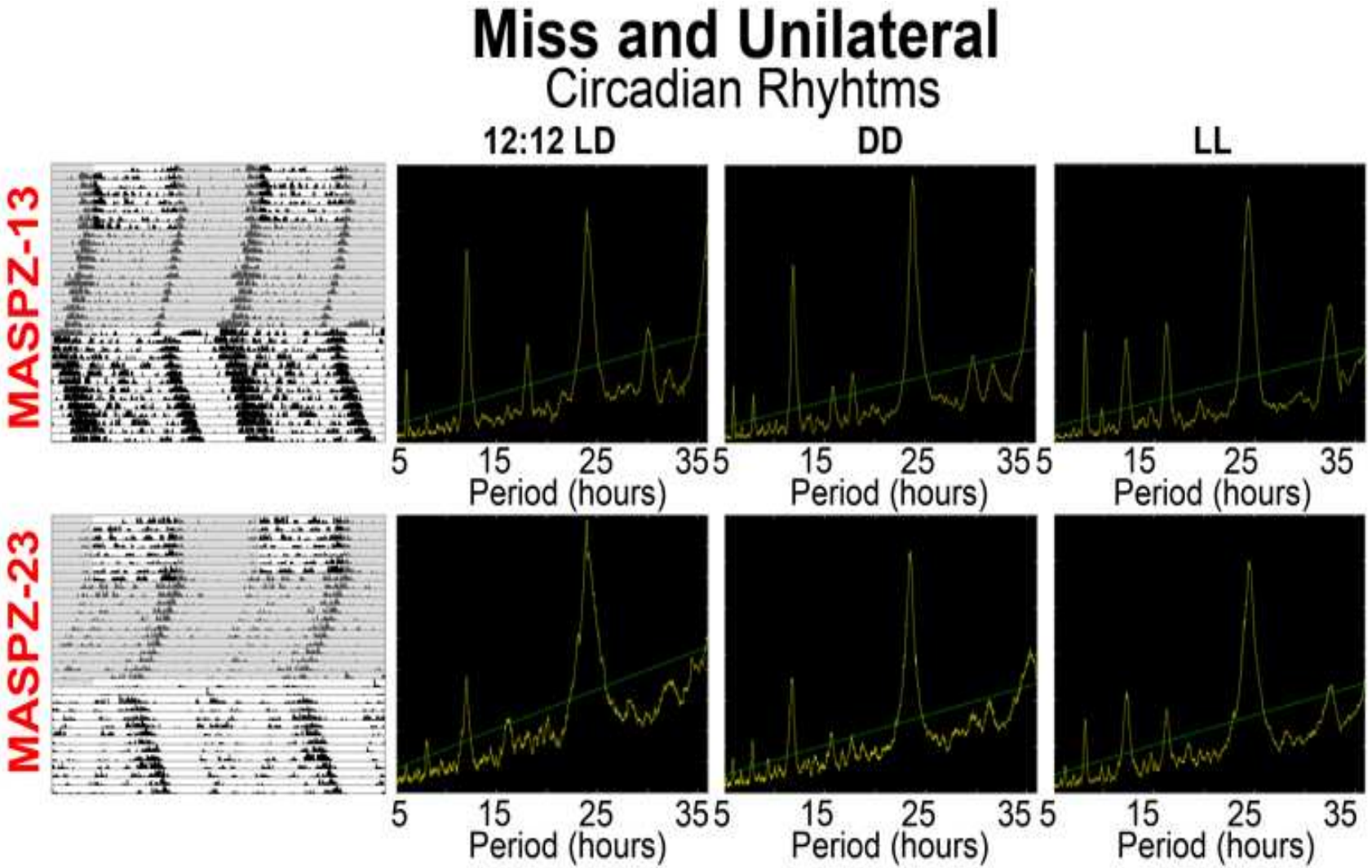


Figure 3.11. (cont'd)

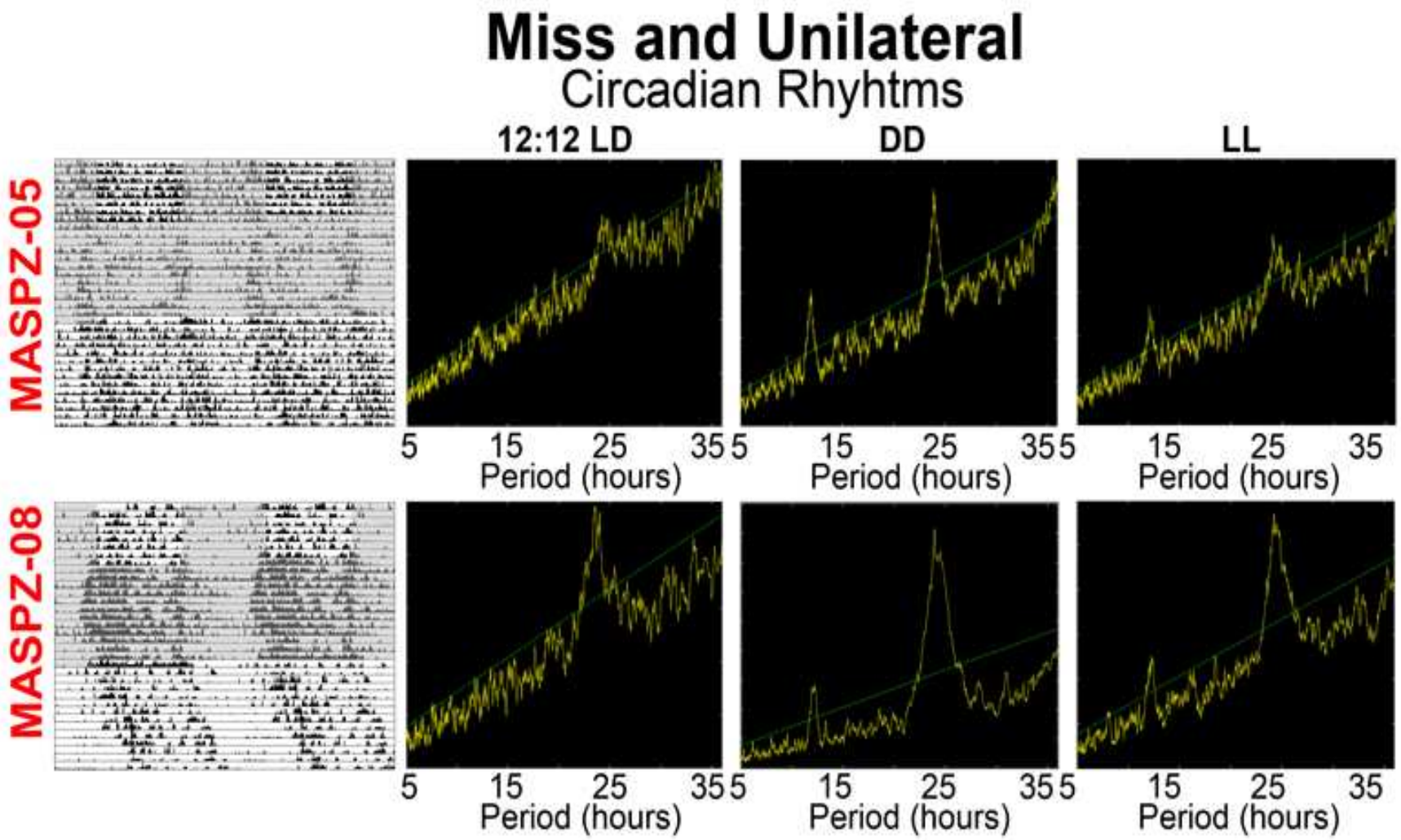


Figure 3.11. (cont'd)

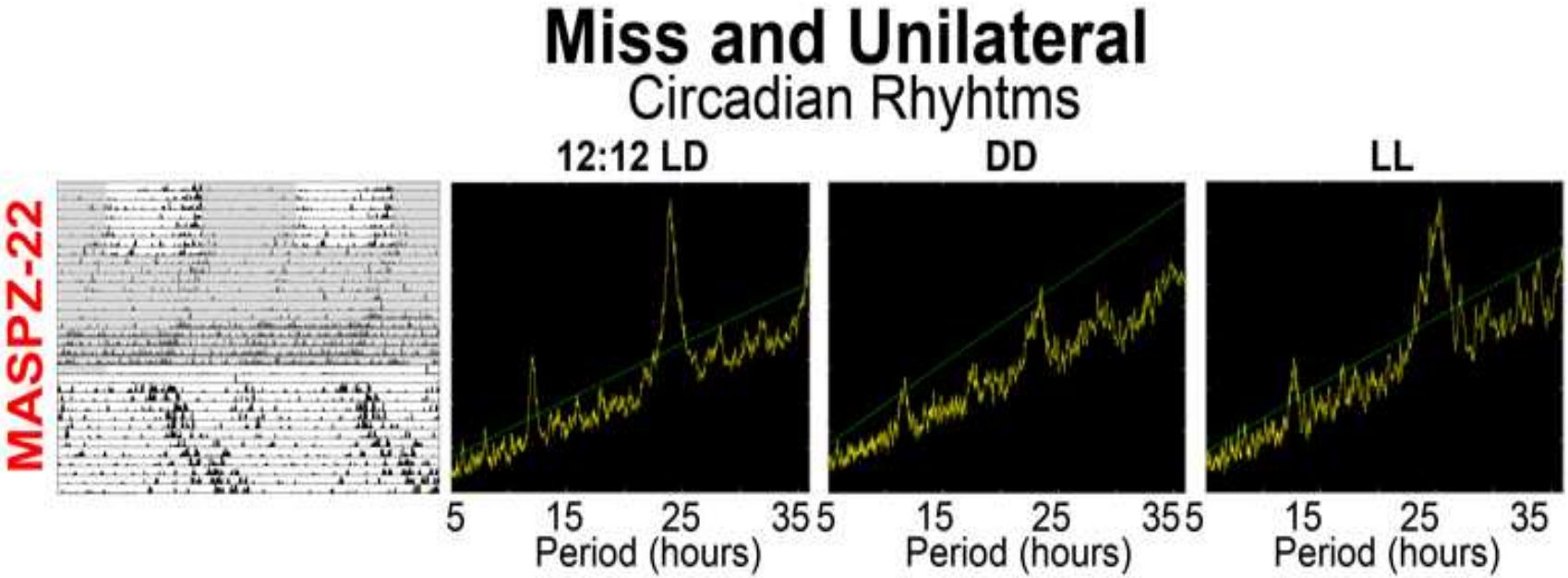
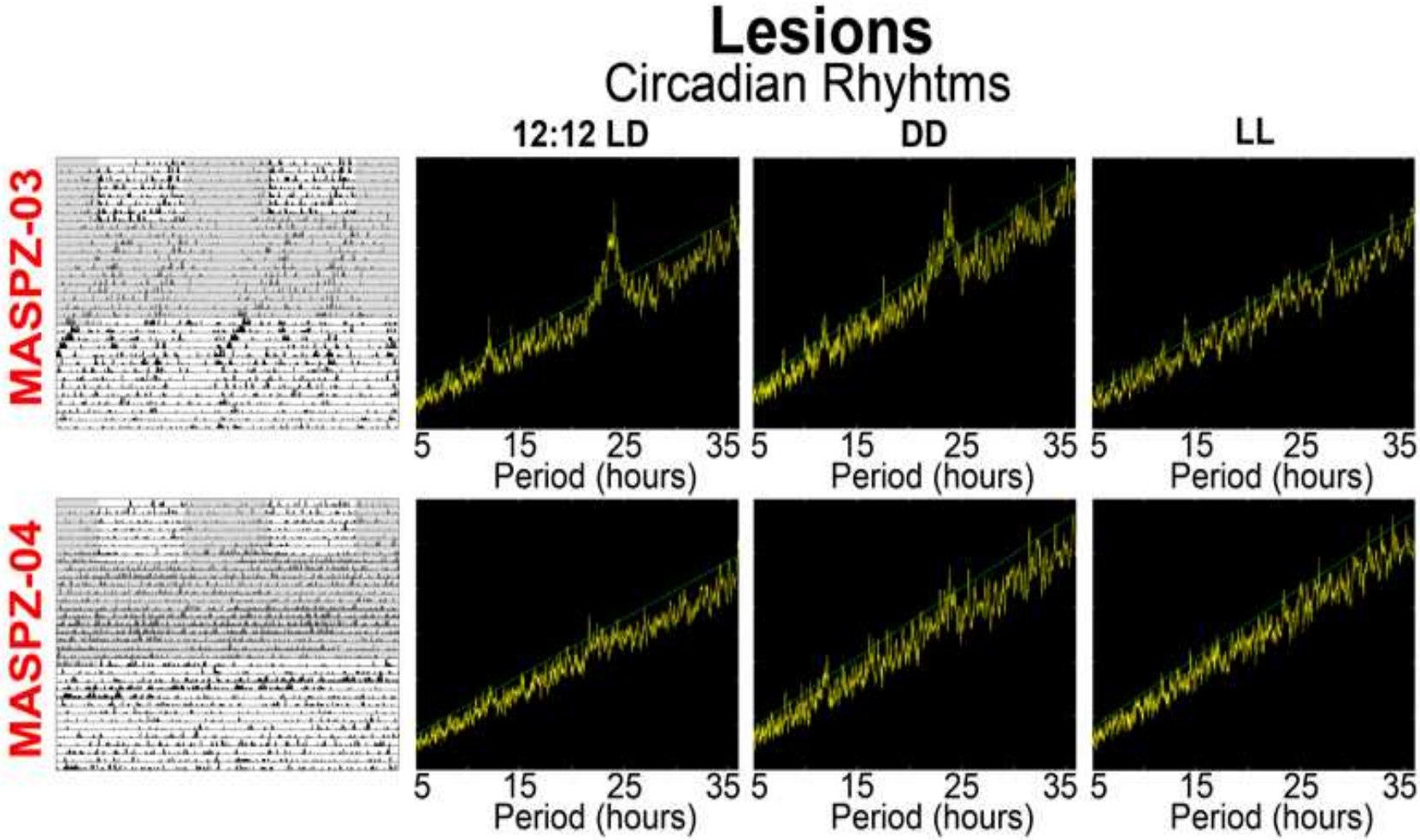


Figure 3.12. Circadian rhythms analysis in varying lighting conditions in Bilateral lesion animals. On the Far left, actograms of all animals across three different lighting conditions, 12:12 LD, constant light, and constant darkness. Chi-squared analysis was conducted to detect the presence of significant rhythms were run for all lighting conditions. One animal had no rhythms at any light conditions (MASPZ-04). In Constant darkness MASPZ-10 became arrhythmic and in constant light MASPZ-03 was no longer rhythmic.



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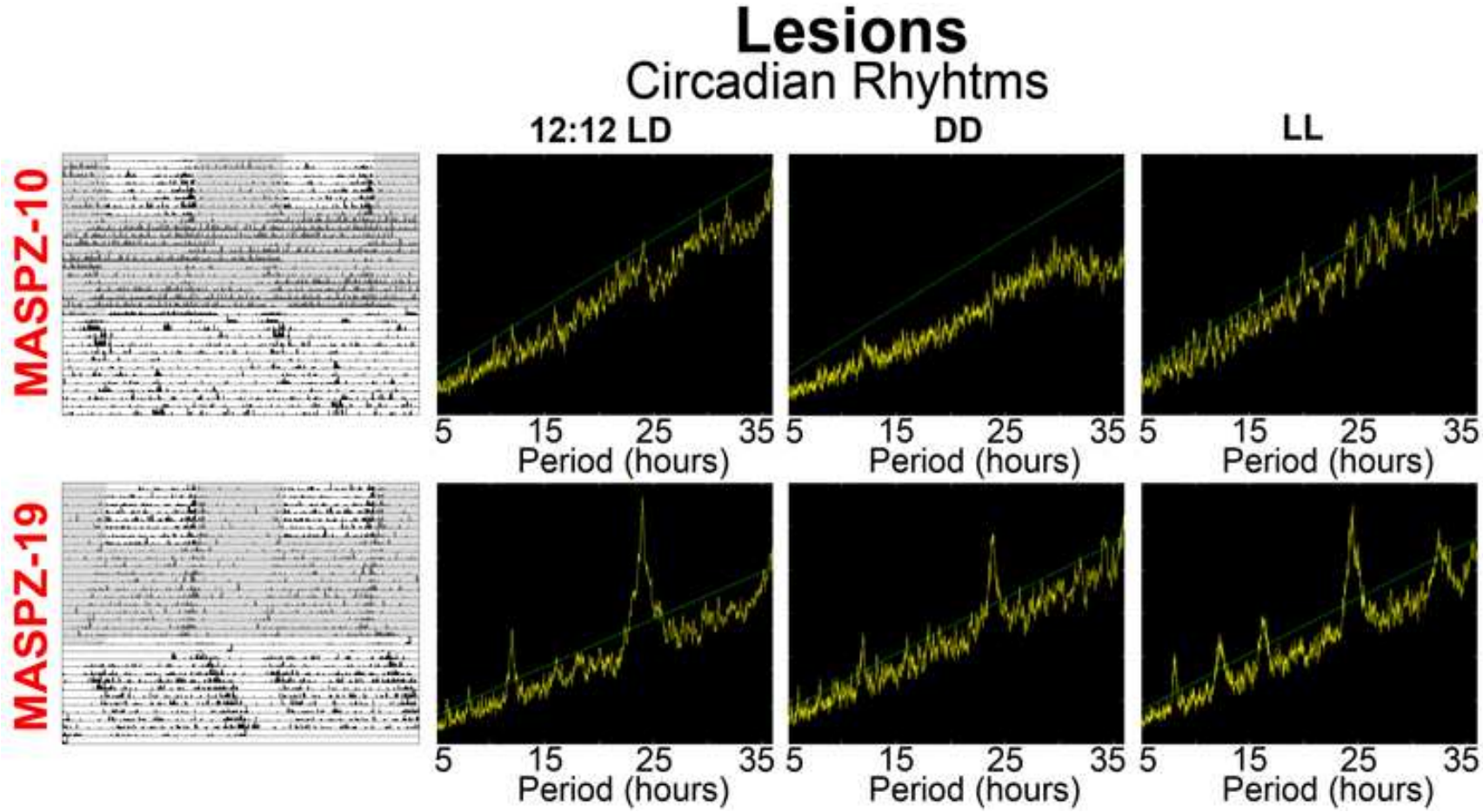


conditions after vSPZ lesion, are still able to mask to a LD cycle. Here too we observed that a majority (3 of 4 animals) of our completely lesioned animals displayed diurnal activity patterns in LD conditions with the exception of the one animal with the greatest SCN damage (MASPZ-04). Altogether, our current data supports the idea that the vSPZ is not crucial for the masking response of grass rats.

Circadian Rhythms

Complete ablation of the vSPZ in our animals greatly reduced the expression of the rest-activity cycles in constant conditions. Previous attempts to lesion this area in the grass rat did not succeed in destroying the entire vSPZ, however, the size of the partial lesions was negatively correlated with both the strength of the rhythms and reentrainment rate. In nocturnal animals, the destruction of the vSPZ eliminates circadian rhythms in activity (Abrahamson & Moore, 2006; Lu et al., 2001) and sleep (Lu et al., 2001) but does not impact temperature rhythms. Here we observed the same diminished expression of circadian rhythms in activity for the grass rats. Most animals (3/4) with complete bilateral lesions of the vSPZ no longer had significant circadian rhythms as detected by chi-squared analysis in constant light. However, one of these arrhythmic animals (MASPZ-04) had the most damaged SCN of all animals in the study. We, therefore, cannot draw conclusions about the effects of vSPZ lesions on circadian rhythms in the grass rat because of our small sample size. More investigation into the role of the vSPZ in the circadian regulation of activity is needed to determine if it plays a similar role in circadian regulation in nocturnal and diurnal animals.

SCN Damage

Two of our 4 animals in which the vSPZ was completely lesioned also had extensive damage to the SCN (Figure 3.3). It thus appears that in the grass rat NMA is an effective agent at high doses for inducing the destruction of the SCN. An earlier study by Schwartz et al. (2009) found that the SCN of grass rats appeared to be intact while the vSPZ was damaged by this chemotoxin, but in the current study we used a substantially higher concentration and volume of NMA. Previous work in nocturnal rodents has suggested that the SCN is resistant to direct injections of chemotoxins, such as NMDA (Hastings, Roberts, & Herbert, 1985) and kainic acid (Schwarcz & Kohler, 1983).The concentration of toxin used by Hastings et al. (1985) was half that used in our experiment, but the volume was almost three times higher; the total amount administered in that study was therefore slightly higher than in this one. The current finding that the SCN of grass rats is vulnerable to chemical lesions may thus reflect the high concentration of the toxin that we used, but it also raises the possibility of a very interesting species difference in SCN function.

Summary and Conclusions

The data described here must be described as preliminary; however they do lead to two clear conclusions: (1) The vSPZ, which had been a prime candidate for the mediation of masking, is not necessary for it in grass rats, and (2) the SCN of these animals is susceptible to chemotoxic effects of NMDA, challenging prevailing views about the resistance of the SCN to such agents.

The hypothesis that the vSPZ plays a role in mediation of differences in masking responses of diurnal and nocturnal species had emerged for several reasons. The region has input from ipRGCs (Hattar et al., 2006), and cells in it undergo an increase in cFOS after light exposure in grass rats, which is not the case in nocturnal mice (Chapter 2). Furthermore, although there are no other experiments that have directly examined the role of the vSPZ in masking, lesions that have damaged both the SCN and the vSPZ have abolished masking in hamsters (Li et al., 2005). Data from the current study, suggest that this is not the case in diurnal grass rats. Although the number of animals with complete lesions was small (n=4), we saw a difference between activity levels in control and LP conditions in these animals, and one whose vSPZ was completely destroyed showed clear evidence of masking in a 7 hr LD cycle. It should also be noted that investigations into the neural mechanisms of masking in nocturnal species has suggested that masking is mediated by several redundant retino-recipient regions (Redlin, 2001). These may operate within a neural network that balances positive and negative influences of light on activity in ways that lead to species patterns of masking. Further tests using gradients of light intensity (Redlin et al., 1999) should therefore be used to determine if the vSPZ plays a supportive in the masking response under other conditions. Although the neural mechanisms of masking remain elusive, the current data have established that the vSPZ of diurnal grass rats is not crucial for its maintenance.

CHAPTER 4

The pineal gland does not influence the daily patterning of behavior of diurnal *Arvicanthis niloticus*

ABSTRACT

Melatonin is a hormone rhythmically secreted by the pineal gland, with peak levels occurring during the night. The hormone in rats and hamsters improves the reentrainment rate of the circadian activity rhythms and alters the masking response to a reversed light:dark (LD) cycle. In diurnal animals, melatonin is present during the inactive phase of the rest/active cycle and in primates, it directly influences sleep and body temperature. Pinealectomized humans also have a higher incidence of sleep disruption after surgery. Few studies have used a non-primate, diurnal mammalian species to examine the role of the pineal in promotion of the patterning of activity and sleep at night, masking responses to light, or basic parameters of the circadian timekeeping system, such as period and phase of the rhythms. Here, the authors directly examined the hypothesis that melatonin contributes to diurnal patterns of behavior and physiology of a diurnal rodent, *Arvicanthis niloticus*, first by administering the hormone to intact animals, and then by removing the pineal gland, the primary producer of the circulating melatonin. Exogenous melatonin ranging from physiological levels to high pharmacological doses did not suppress body temperature or reduce activity when administered at ZT11, a very different response than observed in primates. Removing the pineal gland had no effect on the hourly distribution of activity across a 12:12 h LD cycle, or on the patterns of sleep across the dark phase of that cycle. The direct, masking, effects of light at night on activity were also the same in

pinealectomized and control grass rats, as 1 hr pulses of light across the dark phase of a 24 h LD cycle stimulated increases in activity of control and pinealectomized animals to a similar extent. In addition, the circadian regulation of activity, as indicated by the responses of rhythm period and phase to changes in lighting conditions, was unaffected by the surgical condition of the animals. Taken together, therefore, these data suggest that the pineal gland does not contribute to the expression of diurnality in grass rats, as it does in primates. These data are discussed in light of what they may tell us about the evolution of diurnality in mammals.

INTRODUCTION

Melatonin is a hormone rhythmically produced by the pineal gland that is elevated in both nocturnal and diurnal species during the dark phase of the daily light:dark (LD) cycle and during the subjective night when animals are held in constant conditions (Reiter, 1993). The nighttime rise in melatonin is driven by circadian mechanisms, but its secretion can be directly suppressed by light (Lewy, Wehr, Goodwin, Newsome, & Markey, 1980; Nelson & Takahashi, 1991). Receptors for the hormone are found in many regions of the brain and in peripheral tissues (Ishii, Tanaka, Kobayashi, Kato, & Sakuma, 2009; Slominski, Reiter, Schlabritz-Loutsevitch, Ostrom, & Slominski, 2012; Stankov, Fraschini, & Reiter, 1991). The functions of melatonin are wide ranging, and it appears to play a role in immune function (Carrillo-Vico et al., 2006; Haldar, Rai, & Singh, 2004), neuroprotection (Katona et al., 1998; Mayo, Sainz, Antolin, & Rodriguez, 1999; Mayo et al., 1998), reproduction (Reiter et al., 2009) and rates of disease progression (Baydas, Koz, Tuzcu, & Nedzvetsky, 2008; Otalora, Madrid, Alvarez, Vicente, & Rol, 2008; Xia et al., 2008). Melatonin can also influence the period and phase of internally generated circadian rhythms of many species, both nocturnal and diurnal (Pevet, 2003). In addition, there is evidence that melatonin can influence sleep, activity, and body temperature in a direct, circadian-independent, manner in some species (Badia, Myers, Boecker, Culpepper, & Harsh, 1991; Cajochen, 2007; Zhdanova, 2005).

Activity-suppressing effects of melatonin have been noted in diurnal fish (Lopez-Olmeda, Madrid, & Sanchez-Vazquez, 2006; Zhdanova, Wang, Leclair, & Danilova, 2001), birds (Murakami, Kawano, Nakahara, Nasu, & Shiota, 2001), old world monkeys

(Hao & Rivkees, 2000; Inui & Hazeki, 2010; Zhdanova et al., 2002), and in humans (reviewed in Lavie, 1997). Two studies have directly compared diurnal and nocturnal species. In nocturnal owls and trench, activity is not altered by melatonin exposures, whereas in diurnal sparrows, quail and goldfish activity is decreased (Lopez-Olmeda et al., 2006; Murakami et al., 2001). Many studies have examined the sleep-promoting effects of exogenously administered melatonin in humans (e.g. Dollins et al., 1993; Dollins, Zhdanova, Wurtman, Lynch, & Deng, 1994; Nave, Herer, Haimov, Shlitner, & Lavie, 1996; Zhdanova et al., 1995; Zhdanova, Wurtman, Morabito, Piotrovska, & Lynch, 1996). In his analysis of the published data, (Lavie, 1997) concluded that if melatonin is administered at times of day during which it is not already elevated via endogenous mechanisms, melatonin has a sleep promoting effect, and suggested that its endogenous role is to inhibit wakefulness-generating mechanisms during the night. Further data on the role of the pineal gland in the regulation of daily sleep patterns in humans has come from studies of individuals that experience injuries that interfere with signals to the pineal (Biering-Sorensen & Biering-Sorensen, 2001; Scheer et al., 2006) or have had the organ surgically removed (Kocher, Brun, Borson-Chazot, Gonnaud, & Claustrat, 2006; Petterborg, Thalen, Kjellman, & Wetterberg, 1991). These studies have revealed that such individuals have an increase in sleep dysfunction, though it is impossible to rule out the possibility that other features of their conditions are the source of the problem. However, in some such cases, administration of melatonin improves sleep-related symptoms (Etzioni et al., 1996; Jan, Tai, Hahn, & Rothstein, 2001; Lehmann, Cockerell, & Rudge, 1996). Little is known about direct effects that the pineal may have on sleep and activity in diurnal mammals other than primates.

Another way in which the pineal gland could promote sleep is via an influence on the masking response of animals to light. Masking refers to a process whereby light directly affects behavior, such as the induction of sleep in nocturnal species and wakefulness in diurnal ones. Two studies have revealed elevated masking responses to light in pinealectomized vs. control nocturnal rats (Quay, 1970; Vilaplana, Cambras, & Dieznoguera, 1994). Comparable studies with diurnal species have not been reported, however, treatment with melatonin has been found to alter the masking response of body temperature and sleep to light at night in humans. Young men given an infusion of melatonin and then exposed to bright lights showed a reduction in the light-induced increase of body temperature (BT; Strassman, Qualls, Lisansky, & Peake, 1991) and had shortened latencies to sleep onset (Burgess, Sletten, Savic, Gilbert, & Dawson, 2001) when compared to the controls.

Virtually nothing is known about the role of the pineal gland in regulation of behavior of non-primate diurnal mammals. Here, our primary goal was to examine the hypothesis that the pineal gland contributes to the decrease in activity and increase in sleep that occurs at night in the Nile grass rat, a diurnal murid rodent from East Africa. We did this through examination of the possibilities that it has direct effects on these behaviors and that it might mediate masking responses to light. In addition, we tested whether it modulates the circadian regulation of activity. We did this first by administering melatonin to these animals and monitoring their activity and Tb, and then by determining whether removal of the pineal gland would affect these basic processes.

METHODS

Animals

Adult male and female *Arvicanthis niloticus* (grass rats) from the breeding colony at Michigan State University were singly housed in Plexiglas cages (34x28x17 cm) for the duration of these experiments. The facilities were maintained at a temperature ranging between 20-25°C with a 12:12 light:dark (LD) schedule. Animals were also given food (PMI Nutrition Prolab RMH 2000, Brentwood, MO) and water *ad libitum.* All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University and were treated in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

Experimental Procedures

Experiment 1: Effects of exogenous melatonin on activity and body temperature

Six adult male grass rats were surgically implanted with E-mitters (PDT ER-4000 G e-mitters, Minimitter, Bend, OR) to monitor body temperature and general activity, following the protocol outlined by (Schrader et al., 2009). In brief, grass rats were first anesthetized using isoflorane (2.5-5%, Abbott Laboratories, IL, USA) and then injected s.c. with buprenorphine hydrochloride (Buprenex, 0.06 mg/kg of body weight) prior to the surgery. Along the midline of the peritoneal cavity we made a 1 cm incision and the transmitter was placed into the cavity in a position underneath the liver. The abdominal muscle was then closed with nylon suture and the skin was secured with dissolvable chromic gut sutures, which were then reinforced with autoclips. At the conclusion of the surgery, animals were injected with sterile saline (2 cc, 0.9% NaCl) and Ketoprofen

(5mg/kg of body weight). Animals received the analgesic Melaxicam (0.1 mg/kg of body weight) orally in apple every 24 hrs for the next 2 days.

After a recovery period of at least two weeks, animals were administered varying doses of melatonin (0.33 mg/kg, 1.0 mg/kg, 4 mg/kg, 5 mg/kg of body weight) or a vehicle control (ethanol) in a piece of apple, using a protocol similar to that described in Hiebert et al. (2006). Melatonin (Sigma Chemical, St. Louis, MO) was first dissolved in 100% ethanol to an appropriate concentration that allowed for 10-25 μL of solution to be administered, based on the weight of the animal. Solutions were made on the day of exposure and stored at 4°C in a 2 mL microcentrifuge tube shielded from direct light by foil until use. A 5 mm^3 piece of apple was injected with the solutions using a Hamilton syringe and then the ethanol was allowed to evaporate for at least 30 minutes before being fed to the animals. Grass rats were habituated to receiving apple prior to the experiment; animals always took samples voluntarily and consumed them immediately. Each animal was exposed to the control and then the experimental conditions from the lowest-to-highest dose in a within-subjects design; each exposure was administered at least 4 days after the previous one.

Experiment 2: Effects of pinealectomy on activity and sleep behavior

Twenty adult female grass rats were set up with infrared motion detectors (IRs, Visonic Tel Aviv, Israel) to monitor general activity. Animals were randomly assigned to either the pinealectomy (n=10, PINX) or control (n=8, SHAM) group. All animals were anesthetized using isoflorane and then the surface of the head were shaved and sterilized with betadine. Animals were secured in a steretaxic apparatus (Stoelting Co.;

Wood Dale, IL) and then injected s.c. with Buprenex and lidocaine (0.03 mL) prior to the surgery. A 2 cm incision was made on the scalp to expose the surface of the skull. Above Lambda, a circular hole was made with a 5 mm trephine and, using fine forceps, the pineal gland was quickly extracted. Two researchers examined the extracted gland to confirm that it had the distinct texture and shape of the pineal; samples were also stored in PBS and later examined by D.D.S. under a dissecting scope to further confirm that the pineal gland had been removed. The hole in the skull was packed with gel foam and the incision was closed with autoclips. Post-operative care was the same as described above. SHAM animals followed the same surgical protocol without the removal of the pineal gland.

After a recovery period of at least two weeks, animals went through multiple tests to examine patterns of activity and sleep as well as responses to changes in lighting conditions. First, grass rats were monitored in 12:12 LD cycle for 1 week. The animals were then placed into constant darkness (DD) for 2 weeks and then transferred into constant light (LL) for 2 weeks (Figure 4.5A). Animals were then placed in a 12:12 LD cycle until they all displayed stable entrainment. At this point, over an eight-day period, each animal was videotaped across the dark phase of the LD cycle with two low-light lens CCTV cameras connected to a time-lapse video recorder that condensed a 12 hr period onto a 2 hr tape. These tapes were then converted into DVDs that could be seen on a computer screen and the behavior was scored with a custom-made data acquisition program (S & K Computer Products, Toronto, Ontario, Canada; Lonstein & Stern, 1997). The details of the scoring are described below. Animals were then exposed to a masking protocol in a 12:12 LD cycle (Shuboni et al., 2012). Specifically,

animals went through a series of three day cycles during which they were exposed to a 1 hr light pulse (LP) or dark pulse (DP) on the third day; DPs were administered first (at ZT2, 10, and 6) and then LPs (at ZT14, 22, and 18). Finally, grass rats were phase shifted to determine the rate of reentrainment after a 6 hr phase delay and then a 6 hr phase advance. At the end of the experiments blood samples were collected and analyzed to determine melatonin concentrations (described below).

Measurement of Plasma Melatonin Levels

Animals were anesthetized using intraperitoneal injections of sodium penabarital (400mg/kg), and blood samples were collected with heparinized 5cc syringes via cardiac puncture. Each sample was alliquoted into a 2 mL microcentrifuge tubes and spun down in a centrifuge at 1,000 g at 4℃ for 20 minutes. Plasma was transferred into new microcentrifuge tubes and stored at -20℃. Plas ma and quality controlled samples were analyzed for melatonin concentrations in duplicate using a commercial ELISA kit (GenWay Biotech, San Diego, CA) according to the manufacturer's protocol utilizing the competition principle and microtiter plate separation. Six standards and 2 controls included in the kit were used for comparison. A standard curve was obtained by plotting the optical densities (ODs) of the standards (y-axis, linear) against their provided melatonin concentrations (x-axis, logarithmic) in a regression equation using a Hillslope plot with the program, SigmaPlot (Systat, San Jose, CA). The concentration of each unknown sample was then calculated using the equation generated by the standards.

Data Analysis

Experiment 1: Effects of exogenous melatonin on activity and body temperature

Signals from the transmitters interfaced with receiver (ER-4000, Minimiter, Bend, OR) on which each animal's cage was placed. Each base was connected to a computer in an adjacent room, where the data were collected in one-minute bins using Vitalview 4.0 Software (Minimiter, Bend, OR). General activity was recorded by summing counts (movements) across each one-minute period, while body temperature was taken with a single recording at the end of the sampling interval. Data were imported into Microsoft Excel, were all counts were summed into 1 hr samples for activity or averaged for each 1 hr interval for temperature. Two within-subjects one-way ANOVAs were conducted separately for both general activity and body temperature to compare the reaction of animals at ZT11 to varying concentrations of melatonin.

Experiment 2: Effects of pinealectomy on activity and sleep behavior

The IR detectors were attached to the lids of the cages, near the food and water. Each monitor sent information about the motion of an animal (infrared beams breaks) to a personal computer in an adjacent room equipped with the VitalView Program (Minimitter, Bend, OR, USA). To analyze different parameters of the circadian rhythms files were converted into actograms via ClockLab (Actimetrics, Wilmette, IL, USA). The program automatically determined onset and offset of activity, and calculated the period of the rhythm, tau (τ). The experimenter visually inspected these points prior to analysis, as the program occasionally improperly designated onset/offset, and manually corrected errors (as in Schrader et al., 2009). From the onsets and offsets we also calculated the duration of the active period of the daily/circadian rhythms, alpha (α). Using Microsoft

Excel we also examined the raw activity data and determined the total activity per cycle, based on the circadian period in DD, LL, and LD. Additionally, for 5 days in LD we calculated the activity profile in 1 hr-bins across 24 hrs. Finally, two experimenters independently examined the actograms to determine when rhythms had reentrained after phase shifts of the LD cycle; this point was defined as the first day of a period of at least three days during which the time of activity onsets were consistent (e.g. Jechura, Mahoney, Stimpson, & Lee, 2006). Results were all analyzed with mixed two-way ANOVAs. Specifically, for analyses of alpha and total activity the within-subjects variable was lighting condition (DD, LL, and LD) and the between-subjects variable was surgical condition (PINX and SHAM). For tau, we also did a two-way ANOVA; here the within subjects variable was lighting conditions (DD and LL) and the between subjects variable was again surgical condition (PINX and SHAM). Finally, we used independent-sample t-tests to determine the differences between PINX and SHAM animals for both phase delays and phase advances.

"Sleep-like behavior" (referred to here as "sleep") was scored from videotapes when the animal adopted a distinct posture, lying down in a slumped position with the head on the floor of the cage (Figure 4.3A). An observer blind to the condition of the animal recorded onsets and offsets of each sleep bout across the night (using the custom-made data scoring program described above). From these data we calculated the total amount of sleep, the number of sleep bouts, and the duration of those bouts, during the 12 hr period of recording. For analysis of the total sleep bout number and average bout duration, we used independent sample t-tests to compare PINX and

SHAM groups. To analyze the effect of surgery on the amount of sleep per hour across the 12 hr period of darkness we used a mixed two-way ANOVA (Time X Surgery).

To analyze the masking data, we used Microsoft Excel to sum the total amount of activity during the 1 hr of each of the 6 pulses, and the same h on the corresponding control day. For each pulse, we used a mixed two-way ANOVA to compare the pulse condition (dark/light pulse and control) and the surgical conditions (PINX and SHAM). Finally, we compared the absorbance level of the melatonin assay between the PINX and SHAM group using an independent samples t-test.

RESULTS

Experiment 1: Effects of exogenous melatonin on activity and body temperature

General activity was not affected by melatonin during the hour of its administration (Figure 4.1A ; $F[4, 16] = 0.605$, $p = 0.623$). Additionally, we did not observe any alteration in profile of activity when we examined the behavior of the animals across the day after the exposures (Figure 4.1B). There was a near-significant effect of melatonin on body temperature (Figure 4.1C; $F[4, 16] = 4.982$, $p = 0.060$). However, when we examined the 24 hr profile of activity we observed a lower temperature rhythm during one of the exposures (Figure 4.1D). When we corrected for average body temperature across the day, there was no longer a trend toward significance ($F[4, 16] = 1.649$, $p = 0.265$).

Experiment 2: Effects of pinealectomy on activity and sleep behavior

LD Conditions

Figure 4.1. The effects of exogenous melatonin on activity and body temperature (BT). Group data of the hour after administration of melatonin does display alteration of activity (A) nor BT (C) at any concentration level, ranging from 0.00-5.00 mg/kg. When Individuals are plotted across 24 hr none display an alteration in activity (B) or BT (D). The dotted red line at ZT11 indicates the administration of melatonin.

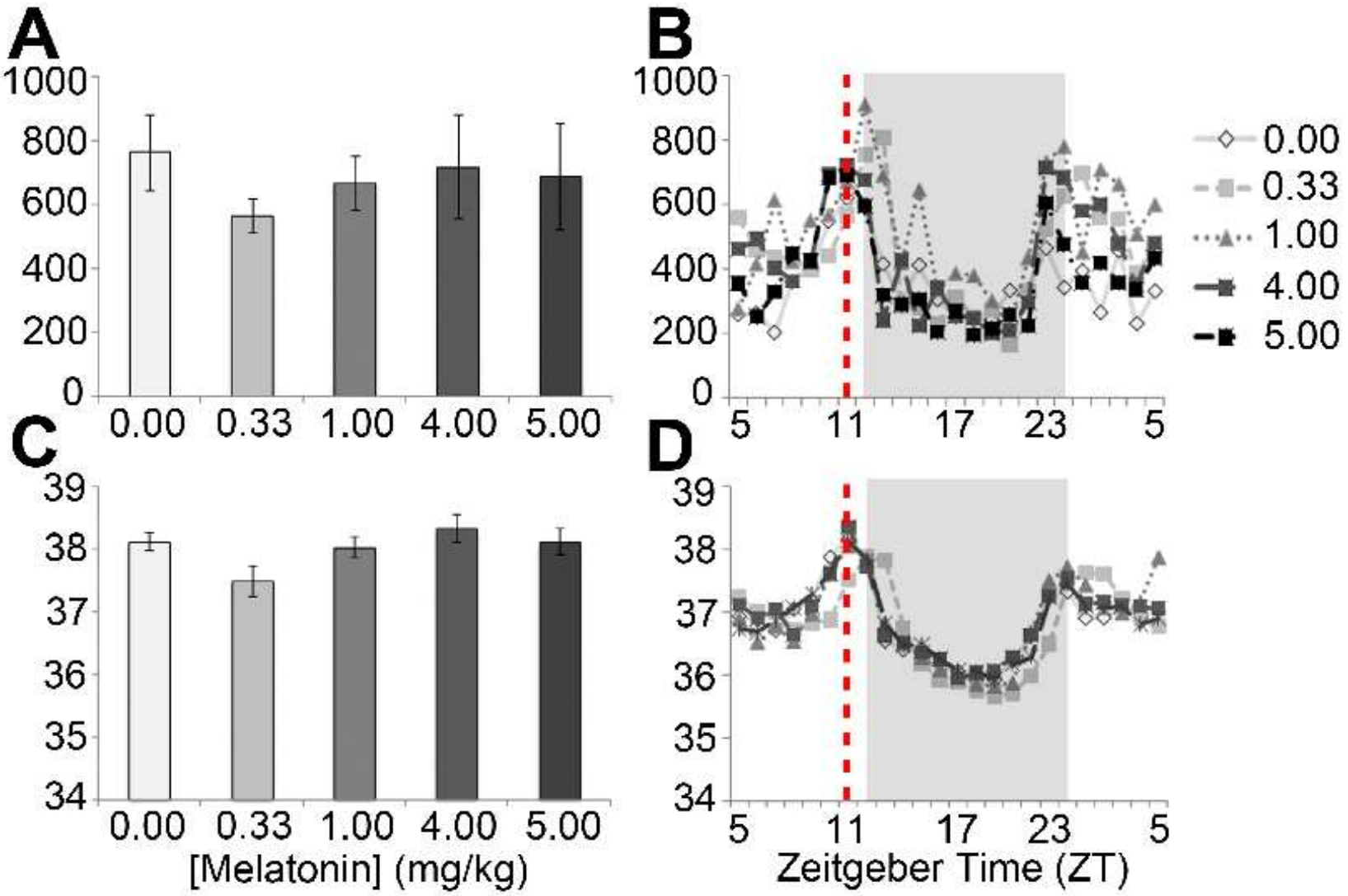
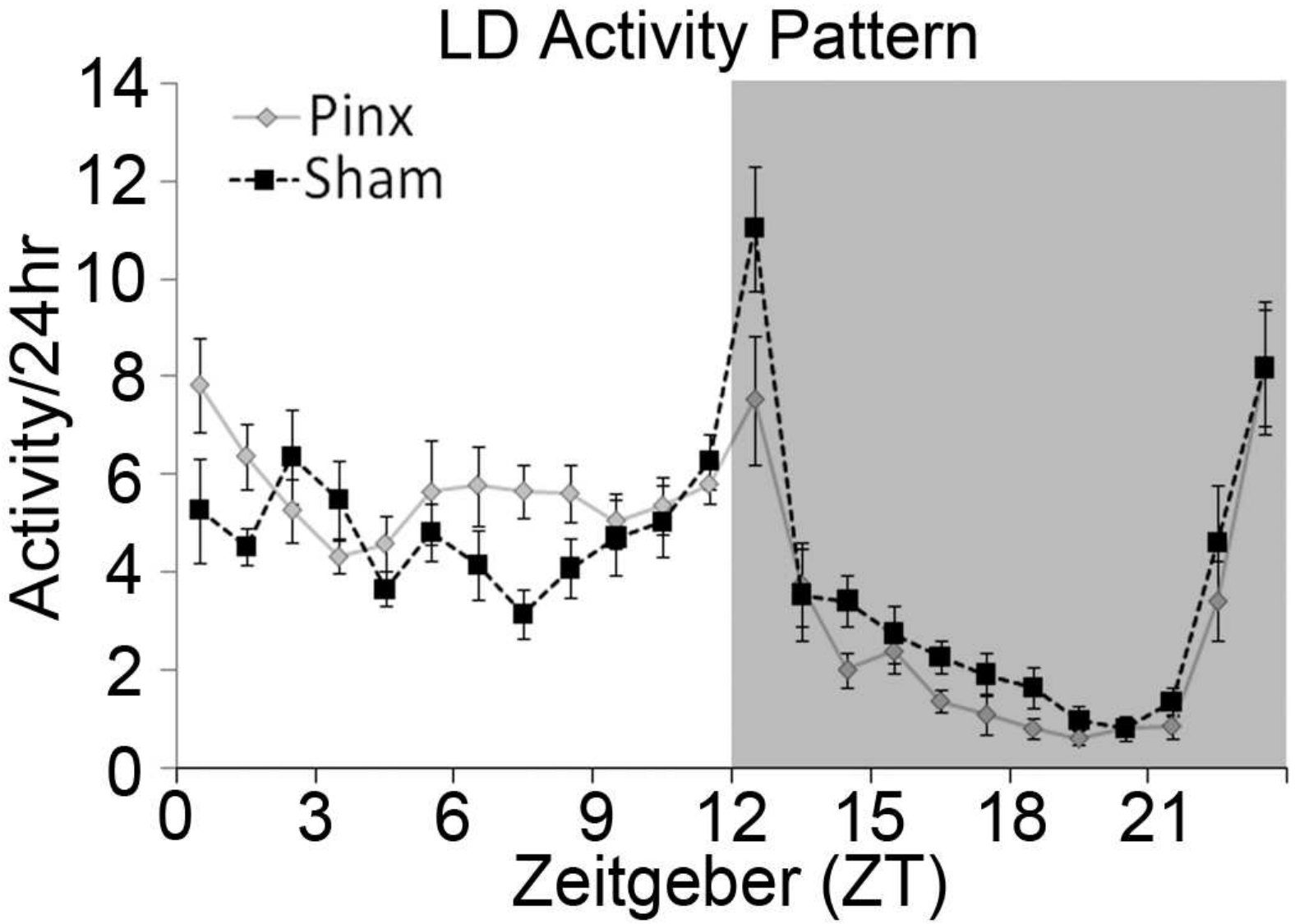


Figure 4.2. 24 hr activity profile of PINX and SHAM animals. Grey bar indicates lights off at ZT12.



In LD conditions, there was a main effect of time on hourly rates of activity (F[23, 345] = 17.954, p < 0.001), but there was no significant effect of surgical condition (F[1, 15] = 0.194, p = 0.666), and there was no interaction between these two variables (F[23, 345] = 2.068, p = 0.085). In both groups of animals the highest levels of activity occurred during the 12 hr light period and at the transitions between light and dark phases of the LD cycle (Figure 4.2B). Under these same lighting conditions sleep bout numbers (Figure 4.3B) and durations (Figure 4.3C) also did not differ in PINX and SHAM groups (t(12)=1.416, p=0.182 and t(12)=0.364, p=0.727, respectively). Hourly rates of sleep across the night were (Figure 4.3D) affected by time (F[11, 132] = 8.401, p < 0.001) but not by surgical condition (F[1, 12] = 0.007, p = 0.934), nor were they affected by an interaction between these two variables (F[11, 132] = 0.361, p = 0.862).

Masking

The masking responses to light did not differ significantly as a function of surgical condition; this was the case for both dark pulses and light pulses (Table 4.1). Among both PINX and control grass rats activity increased in response to light at all three times sampled during the dark period; there was no significant effect of dark pulses during the day (Figure 4.4). The duration of the active period in different lighting periods can also be an indicator of masking, and this too was unaffected by removal of the pineal gland. Specifically, alpha was affected by lighting condition (F[2, 26] = 30.318, p < 0.001) but not by surgery (F[1, 13] = 0.000, p = 0.993), or by an interaction between lighting and surgery (F[2, 26] = 0.379, p = 0.636). Alpha was significantly longer in LL than both LD and DD (Figure 4.5B).

Table 4.1. Analysis of light pulse data masking data in PINX and SHAM animals

Light Pulse			
Time	**ME(Pulse)**	**ME(Surgery)**	**I(PulseXSurgery)**
ZT14	$F[1, 15] = 36.783$ ($p < 0.001$)	$F[1, 15] = 0.057$ ($p = 0.814$)	$F[1, 15] = 0.138$ ($p = 0.715$)
ZT18	$F[1, 15] = 12.541$ *($p < 0.001$)*	$F[1, 15] = 1.761$ *($p = 0.203$)*	$F[1, 15] = 1.759$ ($p = 0.203$)
ZT22	$F[1, 15] = 31.605$ ($p < 0.001$)	$F[1, 15] = 0.776$ ($p = 0.391$)	$F[1, 15] = 0.007$ ($p = 0.936$)

Figure 4.3. Sleep patterns in pinealectomized animals. Panel A demonstrates the behavior we classified as “sleep-like,” the animals adopted a distinct posture, lying down in a slumped position with the head on the floor of the cage. The total number of sleep bouts (B) and the average sleep bout duration (C) were not different between PINX and SHAM animals. Additionally, the spread of total sleep amount across the night was identical between the two groups.

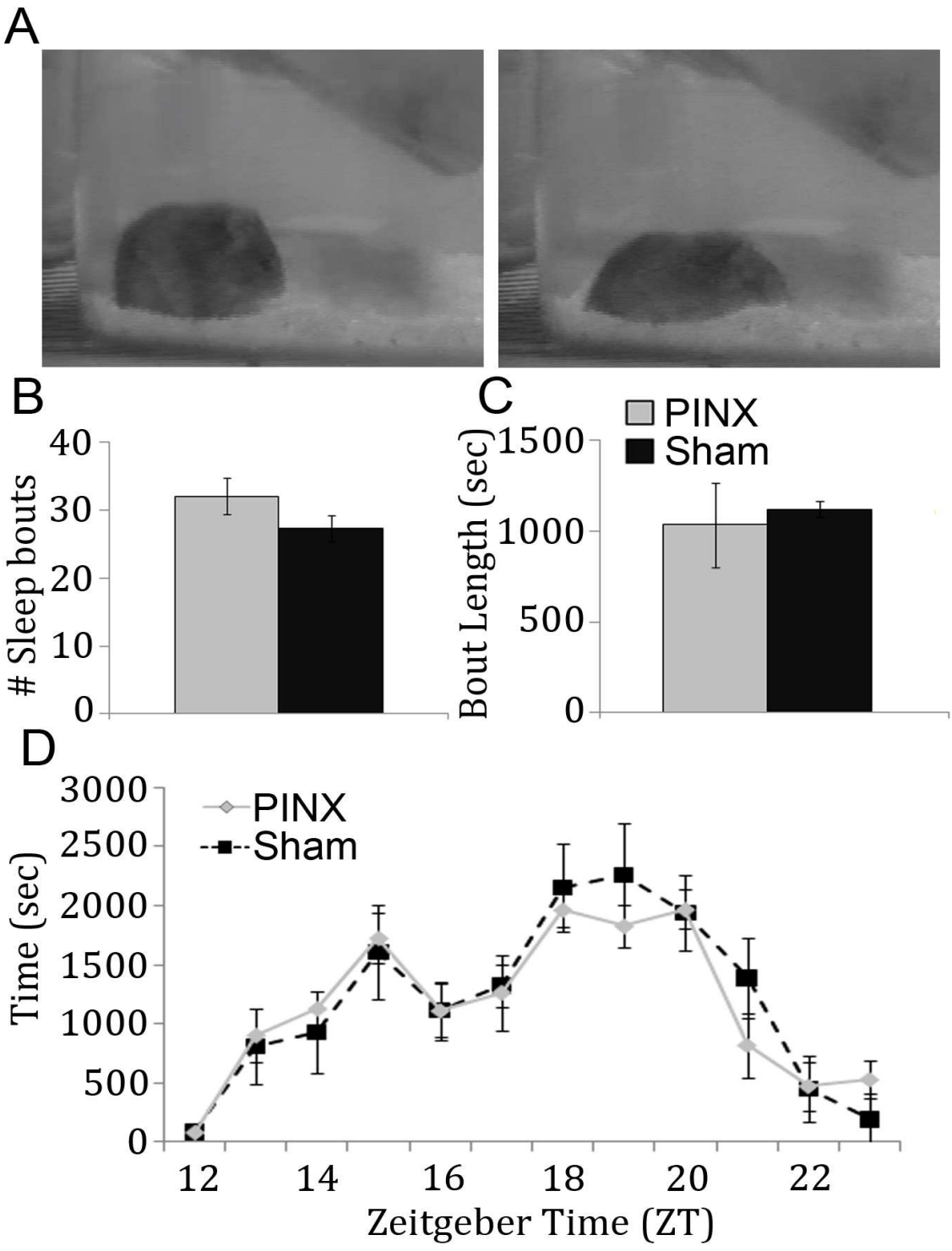
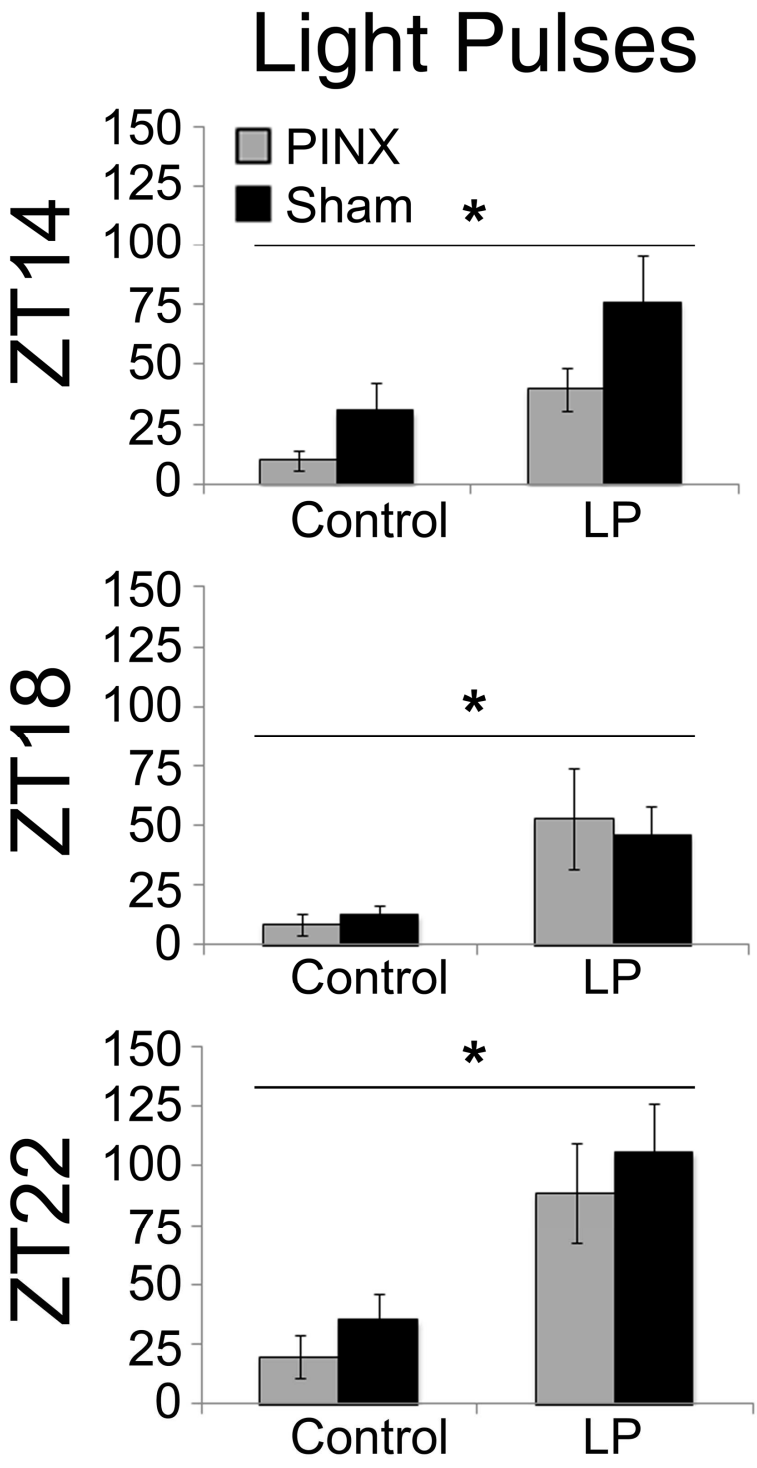


Figure 4.4. Patterns of the masking responses of activity to light. Both SHAM (Black) and PINX (Grey) animals experienced increases in activity at all time points, ZT14, ZT18 and ZT22. An asterisk (*) indicates significance $p < .05$.



Period and Phase Shifts

Tau did not differ in PINX and SHAM groups (F[1, 14] = 1.808, p = 0.200), and there was no interaction between lighting condition and surgery (F[1, 14] = 0.114, p = 0.740). There was a main effect of lighting condition (F[1, 14] = 90.789, p < 0.001) such that the period of the rhythm was significantly longer in LL than in DD (Figure 4.5C). Finally, animals reentrained from phase delays significantly faster than phase advances (Figure 4.6C; F[1, 11] = 5.542, p = 0.038), but, again there was no effect of surgery (F[1, 11] = 0.188, p = 0.673) nor was there an interaction (F[1, 11] = 0.009, p = 0.927).

Melatonin Levels

At the completion of the behavioral experiments, plasma melatonin levels were compared between PINX and SHAM animals. The standard curve created from the set standards provided by the company had an R squared value of 0.9954. One animal was removed from the analysis because of a large difference between the duplicate samples, with a Confidence Variance percentage greater than 25%. Animals without pineal glands had a lower mean level of plasma melatonin, 19.299±2.252 pg/mL, than the SHAMS, 37.565±9.382 pg/mL. When we compared the absorbance levels of the two groups, PINX animals had significantly lower levels than SHAM (t(8)=2.423, p=0.042).

DISCUSSION

Figure 4.5. Circadian rhythms of activity in 12:12 LD cycle, constant light (LL), and constant darkness (DD). Panel A shows two representative actograms of PINX (right) and SHAM (left) animals. There was no significant difference between the two groups for measurement of alpha (B) or tau (C).

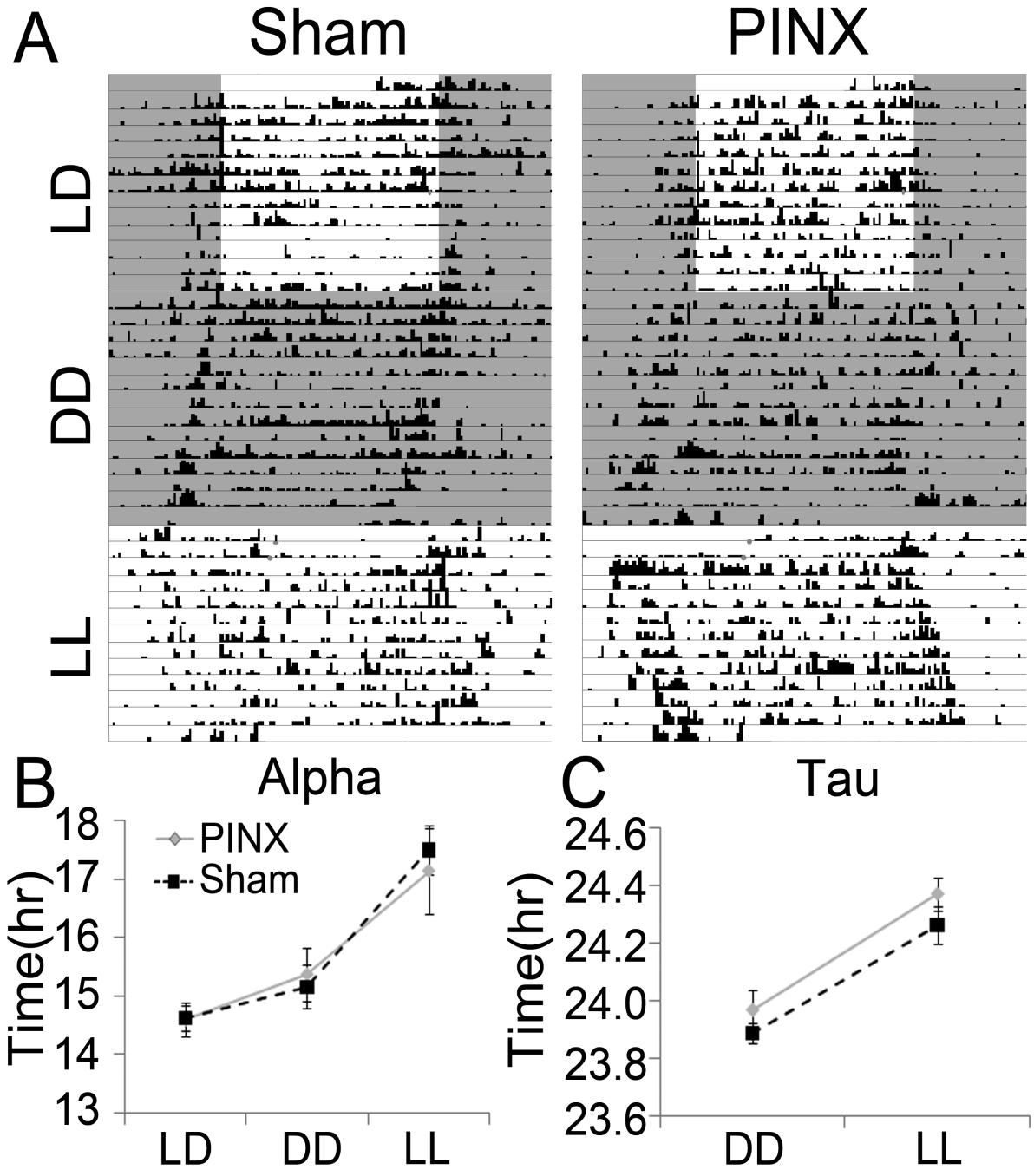
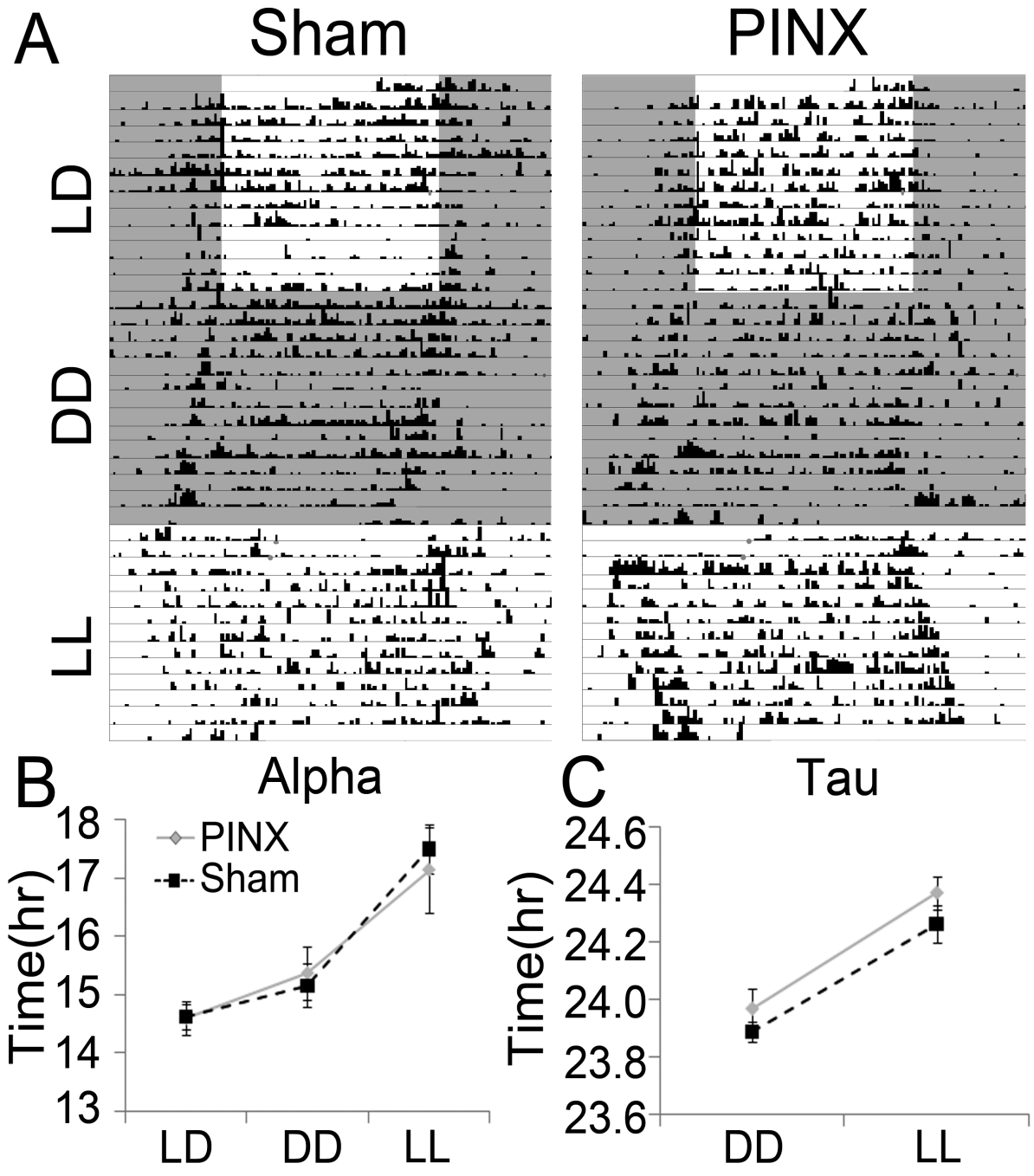


Figure 4.6. Phase shifting ability of pinealectomized animals. Panel A and B are representative of PINX and SHAM animals in response to phase advances and delays. No significant differences were observed between the two groups (C).



The collection of conditions in which we compared intact and pinealectomized grass rats with respect to many features of behavioral rhythms under a variety of lighting conditions, strongly suggest that melatonin does not contribute to diurnality in these animals as it does in primates. Whereas there is good evidence that melatonin can help reduce activity and consolidate sleep at night in primates, this does not appear to be the case in the grass rat. This conclusion stems from data, discussed below, that were obtained in the context of a 24 h LD cycle, a masking paradigm, shifts in a light dark cycle as well as constant lighting conditions.

LD Conditions

Temporal patterns of general activity and sleep in an LD cycle did not differ between pinealectomized and control groups. This was the case for the average overall amount of activity across the day as well as the waveform of the activity pattern across the 24 h LD cycle (Figure 4.2). Furthermore, there was no indication that removal of the pineal gland affected sleep. Video analysis revealed no difference in the overall temporal distribution of sleep across the dark phase or the duration or number of sleep bouts during that time. It should be noted that studies of sleep in pinealectomized nocturnal rodents have yielded contradictory results (Fisher & Sugden, 2010; Mendelson & Bergmann, 2001; Mouret, Coindet, & Chouvet, 1974; Wang et al., 2003). However, there is some evidence that humans may be different in this regard. There are data suggesting that people without a functional pineal gland have sleep that is less consolidated and that extends into the day considerably more than it does in people with normal patterns of melatonin secretion (Macchi & Bruce, 2004; Slawik et al., 2012).

Though most of this information comes from case studies (Macchi & Bruce, 2004), it does suggest that the pineal contributes to consolidation of human sleep at night, presumably through secretion of its hormone melatonin, which has clear soporific effects in other diurnal primates, birds and fish (Hao & Rivkees, 2000; Hughes & Badia, 1997; Inui & Hazeki, 2010; Lopez-Olmeda et al., 2006; Murakami et al., 2001; Zhdanova, 2005; Zhdanova et al., 2002; Zhdanova et al., 2001). Although the pineal may contribute to diurnality in those animals, the current data suggest that this is not the case in grass rats.

Masking

In intact diurnal mammals, light pulses at night directly suppress melatonin and stimulate an increase in activity (Redlin, 2001), raising the possibility that the light induced decrease in melatonin could contribute to positive masking. If that is the case, then pinealectomy should decrease the masking responses to photic stimuli. However, this did not appear to be the case, as we found 1 hr light pulses during the D phase of a 12:12 LD cycle increased activity in both groups and that the magnitude of the response did not differ between the two groups. The stimulatory effect of light at night on activity is thus unlikely to be facilitated by a decrease in melatonin, providing further evidence that melatonin does not influence activity/rest state in these animals.

Data from grass rats maintained in constant conditions also suggests that masking was unaffected by pinealectomy. Specifically, the duration of the active period increased when conditions changed from DD to LL (Figure 4.5B), and the increase was identical in intact and PINX animals. The effect of light intensity on alpha is likely to

reflect masking, with light expanding the active period and darkness reducing it in a diurnal mammal.

Period and Phase Shifts

Though our primary purpose has been to determine whether the pineal gland has any direct influence on activity/rest state, we also examined some features of circadian regulation. In some mammals, pineal melatonin has a small influence on tau and on how it responds to changes in lighting conditions, but in most it does not (Aguilar-Roblero & Vega-Gonzalez, 1993; Aschoff et al., 1982; Cassone, 1992; Cheung & McCormack, 1982; Morin, 1993; Morin & Cummings, 1981; Yanovski, Rosenwasser, Levine, & Adler, 1990). The evidence that we obtained by removing the pineal gland suggests that this is also the case in grass rats. We saw no significant difference between PINX and SHAM animals in period, or in its lengthening upon transfer from DD to LL (Figure 4.5C). In another diurnal rodent, the golden mantled ground squirrel, tau changes in response to photic stimuli were similarly unaffected by light intensity(Martinet & Zucker, 1985). Rates of reentrainment were also unaffected by PINX in grass rats (Figure 4.6), which was also the case in ground squirrels (Martinet & Zucker, 1985), but they are in some nocturnal rodents under some conditions (Finkelstein, Baum, & Campbell, 1978; Quay, 1970, 1971).

Melatonin Levels

Circulating melatonin was higher in control than PINX animals, but the magnitude of the difference was not as great as we expected. The fact that some melatonin was

present in PINX grass rats was not surprising, as surgical removal of the pineal does not completely eliminate circulating melatonin in birds (Jin et al., 2011) or rats (Agez et al., 2009). The low levels of melatonin in our intact animals were likely due to the time at which we collected the samples. In a sister species (*Arvicanthis ansorgei)* the decline in production of melatonin occurs between ZT23 and ZT0 (Garidou et al., 2002).

Summary and Conclusions

As noted above, there are numerous reports of soporific effects of melatonin in diurnal vertebrates (for review, Zhdanova & Wurtman, 1997), such as fish (Lopez-Olmeda et al., 2006; Zhdanova et al., 2001) and birds (Murakami et al., 2001). The pineal gland also has sleep-promoting effects in diurnal primates (Hao & Rivkees, 2000; Hughes & Badia, 1997; Inui & Hazeki, 2010; Zhdanova, 2005; Zhdanova et al., 2002), including humans (Matsumoto, 1999; Tzischinsky & Lavie, 1994). There are no data that we are aware of addressing the question of whether the pineal gland might play a direct role in the regulation of activity or sleep in non-primate mammals that have independently evolved a diurnal pattern of adaptation to the day-night cycle. Katz (2011) has argued at a very general level that when common behaviors evolve independently it is likely to occur via similar changes in neural organization, because the potential mechanisms that could produce a given behavioral patterns are limited. In the current study we examined whether this might be the case when it comes to potential changes in the role played by the pineal gland at two independent evolutionary transitions from nocturnality to diurnality, one that occurred among primates and the other within a murid clade. The data here suggests that this is not the case. That is, whereas the pineal

contributes to decreases in activity and increases in sleep at night in diurnal primates, the current data suggest that this is not the case in diurnal grass rats. Further investigation into the distribution of melatonin receptors within diurnal grass rats and humans may provide insight into why melatonin has such a different role. Other, basic mechanisms that promote sleep at night and activity during the day may be the same, but the role that the pineal gland in primates appears to be absent in diurnal grass rats.

CHAPTER 5

Summary and Conclusions

Temporal niche specific responses to light

In the first, introductory chapter of this dissertation, I outline the importance of the masking system for the structuring of daily behavioral rhythms associated with temporal niches. I proposed that during evolutionary transitions between temporal niches, changes must occur in the neural mechanisms that regulate masking of behavior. The results I presented in Chapter 2 indicated that the neural response to light of nocturnal mice and diurnal grass rats is very different, with more areas within the diurnal brain responding to exposure. These differences could be a result of alterations within the input systems that transmit the signal or in how the brain responds to the stimuli.

Previous work in nocturnal rodents has established the importance of melanopsin-containing retinal ganglion cells (ipRGCs) in production of signals for negative masking (Mrosovsky, Salmon, Foster, & McCall, 2000) and as conduits for relaying rod and cone information to induce positive masking (Guler et al., 2008). Some researchers suggest that alterations within this input system are sufficient for inverting rhythms and play a role in temporal niche switches (Mrosovsky & Hattar, 2005). However, these non-visual photoreceptors are also responsible for other functions including the suppression of melatonin and the entrainment of the rhythms within the suprachiasmatic nucleus (SCN), the circadian pacemaker in mammal. Both the pineal and the SCN remain similar after the transition between diurnal and nocturnal species (Challet, 2007). Melatonin levels peak at night in both nocturnal and diurnal species and glutamate rhythms of the SCN are also similar. These similarities also include the peak

in rhythmic expression of clock genes, the transcription-translation feedback loop that is important for generating endogenous rhythms, within both regions (Pineal: Ackermann, Dehghani, Bux, Kauert, & Stehle, 2007; Karolczak, Burbach, Sties, Korf, & Stehle, 2004; SCN: Ramanathan, Nunez, & Smale, 2008). When mice are mutated to alter the input system and display diurnal activity patterns (Doyle, Castrucci, McCall, Provencio, & Menaker, 2006), the pineal and SCN are both altered (Doyle, Yoshikawa, Hillson, & Menaker, 2008). Specifically, mutant animals have later peaks in their rhythms in the clock gene Per1 within SCN cells and peripheral tissue, like the pineal. Alterations in the input systems needed to induce diurnality in a nocturnal rodent, therefore, have broad repercussions that produce animals that are not similar to those found in naturally occurring populations.

However, nothing is definitively known about the input system mediating masking in diurnal species as no direct examination of photoreceptor function has been conducted in a diurnal model. Although, for a human experiencing masking to light, low-spectrum blue light is associated with arousal, i.e. positive masking (Iskra-Golec, Wazna, & Smith, 2012). These intensities are within the activation range of the ipRGCs that produce negative masking in nocturnal animals. The signal carried by these photoreceptors may therefore remain the same, light activating ipRGCs and darkness activating traditional photoreceptors. The difference between nocturnal and diurnal species with respect to their responses to the stimuli probably lies downstream of the ipRGCs, in the neural mechanisms that dictate the masking response.

Neural Mechanisms of Masking

Differences in masking behavior of nocturnal and diurnal species are likely the product of alterations within the neural mechanisms of masking. Literature on nocturnal rodents suggests that there is no singular region that controls the masking response to light (Redlin, 2001); however, several areas have been targeted for investigation into their functional role in the masking response. While the role of the SCN is controversial (Li et al., 2005; Redlin & Mrosovsky, 1999a), lesions of the intergeniculate leaflet (IGL; Redlin et al., 1999) and olivary pretectal nucleus (OPN; Miller et al., 1999) have been shown to alter but not ablate masking of activity and REM induction, respectively. The work in Chapter 2 spurred Gall et al. (2013) to ablate the IGL in diurnal grass rats; here too they observed an alteration and not an ablation in masking after lesion. In the work described in Chapter 3, I further investigated the functional role of one brain area, the ventral subparaventricular zone (vSPZ), which responded differently to light in mice and grass rats, by using directed chemical lesions in the diurnal grass rat. Earlier work in the laboratory suggested that vSPZ lesioned grass rats might be less sensitive to the 12:12 light/dark (LD) cycle, with random bouts of arrhythmia occurring in animals with the largest lesions (Schwartz et al., 2009). We, however, did not observe an alteration in how a small cohort of vSPZ lesioned grass rats responded to light in two different masking protocols. These findings do not eliminate the vSPZ as a possible component of the masking system but they do demonstrate that the region is not necessary for light-induced masking.

Chapter 3 also describes an interesting discovery about the responsiveness of the grass rat SCN to chemotoxins. The SCN was believed to have endogenous protection from toxic agents (Bottum, Poon, Haley, Karmarkar, & Tischkau, 2010).

Unlike other regions, such as the hippocampus, the SCN cells of nocturnal rodents were not damaged by exposure to N-methyl-D- aspartate (NMDA) *in vivo* (Hastings et al., 1985), *in vitro* (Colwell & Levine, 1996) or in cell culture (Bottum et al., 2010). This difference poses the question of why the grass rat SCN responded differently to the toxin when compared to the SCN of nocturnal rodents. It is believed that the SCN of nocturnal and diurnal rodents is very similar (Smale et al., 2003). However, (Novak & Albers, 2002) suggest that the SCN of grass rat is less sensitive to the phase shifting ability of NMDA when compared to nocturnal rodents.

An alternative way to influence the neural mechanisms of masking could be through hormones. The hormone melatonin is produced at night by both nocturnal and diurnal species (Challet, 2007). However, in primates melatonin has a very specific soporific effect not observed in nocturnal animals (Zhdanova & Wurtman, 1997). Melatonin has also been shown to prevent the light-induced increases in body temperature (Strassman et al., 1991) and arousal (Burgess et al., 2001) in humans. Additionally, pinealectomy in nocturnal rodents alters the response of the SCN to light (Gonzalez & Dyball, 2006) and increase negative masking behavior (Quay, 1970; Vilaplana et al., 1994). Pineal derived melatonin may, therefore, play different roles in nocturnal and diurnal animals. In the research described in chapter 4, we removed the pineal gland, effectively eliminating circulating melatonin, and then tested the masking behavior of our diurnal grass rats. Exogenous melatonin presentation did not induce sleep in our grass rats nor did the loss of melatonin after pineal removal alter sleeping patterns. This finding is different from those obtained in research on humans and could be reflective of differences in the pathway for the evolution of diurnality in these two

species. It should also be noted that humans and grass rats have different patterns of melatonin secretion. The onset of melatonin during the night is much earlier for humans than it is for grass rats (Garidou et al., 2002; Wehr, 1991). Masking to light exposure was also not influenced by pineal removal in the grass rat; nocturnal rats are different in this regard. Effects of melatonin on the grass rat are clearly different from those seen in both nocturnal rats and diurnal primates, demonstrating that the hormone is not universally involved in masking behavior.

Implications and Recommendations

Our current data has demonstrated a clear activity pattern dependent responsiveness of the brain to light exposure. Diurnal grass rats had more areas respond to a 1 hr pulse of light when compared to nocturnal mice. This was not due to differences in staining of tissue as both species responded with a strong increase in cFOS activation within the SCN. We propose that the differences between our nocturnal and diurnal rodents are likely the product of alterations within the neural mechanisms of masking and not the input system within the retina. We also established that the vSPZ and pineal gland are not necessary for the masking response to light in grass rats. This research has added to the growing list of regions examined to determine the neural mechanisms of masking.

Understanding the masking system is important because it plays an integral role in how organisms regulate their activity patterns. Beyond the investigation into the evolution of diurnality, masking behavior is also an important contributor to the success of our modern society. As masking has allowed us to overcome the endogenous

circadian clock and to continue to function beyond the natural 24 hr constraints established by the sun. For humans, light stimulates positive masking behaviors by increasing arousal and decreasing sleepiness (Minors & Waterhouse, 1989b). With the invention of high intensity artificial light, people have gained the ability to regulate light exposure and have extended their activity further into the night. This ability to expose oneself to light during the natural rest-phase of the activity cycle has caused problems within our bodies. Specifically, the ability to remain awake through light-induced masking also phase shifts the circadian pacemaker. Chronic inconsistent schedules of light exposures can lead to many different health consequences, including cancer, immune problems and gastrointestinal illness (Erren & Reiter, 2013). In mice, these phase shifts in the circadian system can, over time, lead to the death of older animals (Davidson et al., 2006). Masking, therefore, for humans can lead to detrimental effects because society pushes the system past where it has short-term beneficial effects. Additionally, human light pollution has altered the light landscape for populations of organisms, possibly altering masking behaviors especially within urban communities.

In summary, this dissertation demonstrates that there are clear differences in the masking behavior of nocturnal and diurnal rodents and that these differences are evident within the brain. The neural substrates that coordinate the behaviors are complex and may vary among animals that have taken different evolutionary paths to diurnality, as is demonstrated by the effectiveness of melatonin in humans and not grass rats. However, understanding these systems is important for both humans and wildlife as the environmental landscape continues to change naturally and at the hands

of man. Therefore, further more detailed analysis of the neural mechanisms of masking is merited.

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