SHEDDING LIGHT ON HIPPOCAMPAL FUNCTION

By

Joel Emmanuel Soler

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PUBLIC ABSTRACT

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Environmental lighting conditions have been shown to influence cognitive function in both healthy populations and patients with dementia. However, the underlying neural mechanisms are not well understood. The objective of the work presented is to examine how chronic daytime light conditions impact hippocampal-dependent spatial learning and memory in a diurnal rodent model. The studies tested the hypothesis that light modulates hippocampal function via the hypothalamic orexin system using a combination of behavioral, morphological, pharmacological and molecular approaches. The results shed novel insights into the neural pathways modulating hippocampal functions in a diurnal brain, and have implications into the prevention and slowing down of aging-associated cognitive decline.

ABSTRACT

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In today's modern world, the average person spends most of their time indoors due to the nature of their profession and their associated lifestyle. With artificial lighting being substantially less intense than natural sunlight and seasonal variations of daylight intensity being a significant factor, this leaves the average human being consistently lacking exposure to bright lighting. Environmental lighting conditions have shown to play a significant role in cognitive function in a diverse array of human subjects. Longitudinal studies have found that bright light therapy can improve several aspects of cognition in healthy and clinical populations of varying ages. Moreover, fMRI studies in humans have demonstrated that bright light consisting mainly of shorter wavelengths activates the hippocampus (HPC) at a higher rate than dim or longer wavelength light. However, the neural mechanisms for how light impacts cognitive function is still unclear. To expand our understanding, the experiments within this dissertation attempt to address this knowledge gap by utilizing the diurnal Nile grass rat (*Arvicanthis niloticus*) as a preclinical research model. The grass rat's activity patterns are like that of the average human, with much of its activity being circumscribed to the presence of light (i.e., subjective day) which makes it a suitable animal model for studying light's effect on cognition.

Specifically, the work presented here will look at how light modulates HPC-dependent learning and memory. In the first set of experiments, the levels of long-term daylight illumination were associated with the retention of a spatial navigational task known as the Morris Water Maze (MWM). Grass rats that were housed for four weeks in a 12:12hr bright light-dark

(brLD) cycle exhibited superior MWM performance over animals housed in a 12:12hr dim light-dark (dimLD) cycle. Deficits in MWM performance shown by the dimLD group were rescued with subsequent exposure to brLD conditions. Additionally, reduced levels of brain-derived neurotrophic factor (BDNF) and a decrease in CA1 dendritic spines were associated with dimLD conditions. These results suggest that chronic daytime light deficiency impacts HPC-dependent learning and memory by dampening hippocampal synaptic plasticity. Subsequent experiments revealed that HPC-dependent learning and memory deficits were further pronounced in female grass rats. Although morphometric analyses revealed reduced CA1 dendritic spine density, like in males, BDNF expression was not impacted, which suggests that light may modulate hippocampal function in female grass rats through distinct neural pathways.

Previous studies done in grass rats have revealed that dimLD conditions negatively impact the expression orexin-A (OXA) in the hypothalamus. Based on those findings, the last set of experiments tested the hypothesis that in diurnal mammals, light modulates hippocampal function via the orexinergic system. Intranasal administration of OXA to grass rats in dimLD conditions during MWM training revealed optimal MWM performance, which suggests that these deficits were due to reduced OXA input. Viral vector-mediated knockdown of orexin-1 receptors (OX1R) in the hippocampus of grass rats housed in brLD conditions negatively impacted MWM performance. These results suggest that bright lighting supports HPC-dependent learning and memory through enhanced OXA-OX1R signaling within the HPC. Overall, the present work provides a better understanding of the neurobiological underpinnings of light-modulated learning and memory by identifying and examining molecular pathways linked to synaptic plasticity.

Dedicated to Zuania, Gloria, Yoel, Luis, along with Sparky, Perry, Bubbles, and Waffles. Thank you for the support and the belief in me to carry out this journey of self-improvement, you all played a huge role in this. Additionally, to all those students pursuing their PhDs: do not let the feeling of self-doubt get the best of you, cultivate discipline and believe in yourself.

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KEY TO ABBREVIATIONS

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
brLD	bright light-dark
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CaMKII	calcium/calmodulin-dependent protein kinase II
CNO	clozapine-N-oxide
DAB	3,3'-Diaminobenzidine
dCA1	dorsal CA1
DG	Dentate gyrus
dHPC	dorsal HPC
dimLD	dim light-dark
DREADDs	Designer receptors exclusively activated by designer drugs
EEG	Electroencephalogram
E-LTP	Early LTP
EM	Electron microscopy
fMRI	functional magnetic resonance imaging
GFP	Green fluorescent protein
HPC	Hippocampus
HSV	Herpes-simplex vírus

IACUC	Institutional Animal Care and Use Committee
IEM	Immuno-electron microscopy
IHC	Immunohistochemistry
IN	Intranasal
ipRGC	Intrinsically photosensitive retinal ganglion cell
LD	Light-dark
LNGFR	Low-affinity nerve growth factor receptor
LTP	Long-term potentiation
L-LTP	Late LTP
LTM	Long-term memory
mBDNF	mature BDNF
MEA	Medial entorhinal area
MWM	Morris Water Maze
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NIF	Non-image-forming
NIH	National Institutes of Health
NMDAR	N-Methyl-D-aspartic acid receptor
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
OX1R	Orexin 1 receptor
OX2R	Orexin 2 receptor
OXA	Orexin-A

OXB	Orexin-B
PFC	Prefrontal cortex
PI_3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLCγ	Phospholipase-Cγ
rAAV	recombinant adeno-associated virus
RHT	Retinohypothalamic tract
RSC	Retrosplenial cortex
SAD	Seasonal Affective Disorder
SC	Scrambled control
SCN	Suprachiasmatic nucleus
shRNA	short hairpin ribonucleic acid
STM	Short-term memory
Thr	Threonine
TrkB	Tropomyosin receptor kinase B
Tyr	Tyrosine
vHPC	ventral HPC
ZT	*Zeitgeber* time

CHAPTER 1:

Introduction

Background

Light is necessary for vision, and it is an essential component that allows humans to carry out a multitude of functions by enabling their most remarkable sense, the visual system, through the activation of photoreceptors [1]. In addition to allowing most mammalian species to perform visual tasks and incorporate information regarding their surroundings, light exerts several non-visual effects that can influence both general physiology and neurobiological functions within an organism. These non-visual, or non-image-forming (NIF), effects range from both acute and long-term effects of circadian rhythmicity that govern several physiological functions (in the brain and periphery), cognitive function, and mood states [2, 3]. An interesting aspect to note is that NIF effects produce responses that are independent of the visual system and light evokes these responses through a separate retinal pathway that will be briefly discussed before proceeding to mention how light impacts cognition.

Several lines of evidence have demonstrated that a distinct photoreceptor system mediates NIF effects of light. Studies conducted in blind human subjects with dysfunctional rods and cones have revealed that blue light (~480nm in wavelength) elicits a maximal pupillary constriction response and suppresses the secretion of melatonin [4, 5]. Non-visual responses to light such as the elevation of core body temperature and heart rate, melatonin suppression, among other measures seem to be most responsive to blue light [6]. In comparison to violet and green light, blue light evokes higher activity from various brain regions that are not associated with visual activity [7]. Meanwhile, classic photoreceptors, such as rods and cones, are most responsive to green light (~550nm), which further strengthens the argument that NIF effects of light are not primarily due to the activity of photoreceptors responsible for phototransduction.

Having initially emerged as an attempt to explain why blind humans and rodent models that lacked functional rods and cones displayed intact circadian photoentrainment [4, 8, 9], the discovery of intrinsically photosensitive retinal ganglion cells (ipRGCs) and the photopigment melanopsin [10, 11] was then attributed to playing a vital role in the NIF effects of light that were previously mentioned. This third set of photoreceptors were also shown to be maximally sensitive to blue wavelength light in a similar fashion to NIF effects [12]. Furthermore, ipRGCs have direct efferent projections to multiple brains regions that include several hypothalamic and thalamic nuclei along with a couple of striatal and brainstem structures [13]. Although ipRGCs do not directly innervate regions such as the hippocampus (HPC) that are vital for episodic learning and memory, there is light-related indirect signaling to the HPC through which the retinohypothalamic tract (RHT) serves as a relay. Therefore, the argument of light being able to impact various neurobiological processes, including cognitive function, through NIF effects is seemingly plausible.

Environmental Variations of Light and its Effects on Cognition

Our environmental lighting conditions never remain constant within 24hours, and it is precisely due to these daily variations of ambient light intensity that most, if not all, organisms exhibit various biological and psychological responses that are constrained to circadian cycles. In addition to daily fluctuations of environmental light, seasonal fluctuations have also resulted in annual adaptations of several physiological and behavioral responses for a variety of species, including humans [14]. Although the research efforts are relatively scarce for humans compared to other species, one of the most well-documented behavioral and physiological responses that experience seasonal variations are related to ingestive behaviors. Empirical measures of blood pressure, cholesterol levels, and caloric intake all increase during fall and winter in humans when

compared to spring and summer [15-17]. Although attributing seasonal variations of food intake-related measures directly to seasonal fluctuations of light may not be the primary factor, it is worth to note the strong, positive correlation between the two.

Another human behavioral response that has been studied extensively with regards to seasonal fluctuations of light intensity is the mood state experienced by a subject. Seasonal mood variations that result in the emergence of depressive symptoms during the fall and winter months and remission during spring and summer has been clinically defined as Seasonal Affective Disorder (SAD) [18]. Even though SAD only exhibits a 1.5%-9% rate of prevalence [19], depending on latitude, a larger portion (~18%) of the general population experiences some degree of mood deterioration during fall and winter that fails to reach clinical threshold (known as subsyndromal SAD) [20]. SAD patients have demonstrated that during the period when symptoms are present, they exhibit deficits in cognitive function that range from increased latencies in completing standardized tasks to higher error rates in working and spatial memory paradigms [21-23]. Upon the remission of their symptoms, most SAD patients display typical cognitive function which provides a compelling argument for seasonal variations of light influencing cognitive processes.

Seasonal responses to cognitive tasks have also been documented in healthy, non-clinical populations. A recent study using young human subjects (~21 years of age) revealed a dichotomy on how different cognitive responses fluctuate across seasons [24]. In this study, participants showed maximum and minimum performance in a sustained attention task during the summer and winter respectively, while maximum and minimum performance in a working memory task was recorded during the fall and spring respectively. Additionally, functional magnetic resonance imaging (fMRI) revealed higher activity within the thalamus and the

amygdala associated with optimal sustained attention, and higher cortical and hippocampal activity in the working memory task. Several other studies have shown that exposure to long-term bright light therapy, a conventional treatment for SAD, in a diverse population of subjects has yielded improvements of cognitive function independently of season. While exposed to long-term bright light during the daytime, elementary schoolchildren demonstrated improved academic performance [25, 26], adult office employees experienced increased working productivity [27], and has even shown to slow down cognitive decline in elderly patients diagnosed with early-stage dementia [28-30]. Although many studies have documented the effects of ambient light on cognition within humans, the neural mechanisms that explain how light modulates cognitive function are still unclear.

A Diurnal Animal Model

There have been numerous research efforts that chronicle the effects of ambient lighting as a modulator for cognition in both clinical and non-clinical human populations. However, an appropriate animal model has yet to be developed for investigating the neurobiological underpinnings behind the direct effects of light on cognition. Most basic and preclinical research involving animal subjects are done using nocturnal rodents that are commercially available, and molecular approaches are based off from knowledge generated using these animals. Because humans are mostly diurnal, we would need to use a diurnal animal model to correctly understand how photic modulation of cognitive processes impacts the human brain. Light can reset and entrain the circadian system in diurnal and nocturnal animals in a similar, if not the same, fashion as evidenced by the suprachiasmatic nucleus (SCN) exhibiting increased activity patterns by the presence of light and lack of light dampens activity in both chronotypes [31, 32]. However, extra-SCN oscillators in various brain regions and behavioral outputs demonstrate rhythms that

are phase-shifted depending on the chronotype of the organism. The most outstanding example of this is the onset of light promoting alertness in diurnal animals while inducing sleep in nocturnal species [33]. Therefore, to dissect either the circadian-dependent or circadian-independent mechanisms of light's effect on cognition we need to use a diurnal animal to obtain findings that have more translational value towards humans.

For the past 25 years, Michigan State University has housed and successfully bred Nile grass rats (*Arvicanthis niloticus*) for their inclusion in several research efforts, and is the only active breeding colony in the United States. Nile grass rats display a predominantly diurnal pattern in the wild, as evidenced by them almost exclusively being caught in traps during the daytime [34]. Their diurnal patterns persisted within laboratory conditions as body temperature, food intake and reproductive behaviors were mostly circumscribed to the light phase of their 12:12hr light-dark (LD) cycle [35, 36]. Additional efforts were conducted to determine their suitability as a laboratory animal; the average gestation period for Nile grass rats is around 23 days, which is comparable to traditional models and breeding pairs can produce litters that range from four to nine pups under captivity [37]. The utilization of diurnal animals within the laboratory setting includes, but is not limited to, the Mongolian gerbil (*Meriones unguiculatus*), the degu (*Octodon degus*), and the Nile grass rat. Of the previously mentioned species, the Nile grass rat displays the highest diurnality index (0.87) with the total duration of its active phase (13.5hrs) being closest to that of humans (15.5hrs) [38, 39]. Although recent findings have demonstrated that the antelope ground squirrel (*Ammospermophilus leucurus*) displays a higher diurnality index (0.97), the African grass rat displays a higher track record of neuroanatomical and neurophysiological research efforts that make it a suitable candidate.

Experimental Lighting Paradigm

In a naturalistic environmental setting, ambient light conditions fluctuate in two ways: overall length of daylight and overall lighting intensity. Seasonal variation of day length is a common occurrence in geographical locations that are further away from the equator, with the most drastic changes occurring during the summer and winter solstices as day length is substantially lengthened and shortened respectively. In consequence, alterations of day length can lead to circadian disruption [40], sleep fragmentation [41], mood disturbances [42] and cognitive dysfunction [43]. However, most humans today are surrounded by artificial lights and technology that can block out light, so drastic changes in photoperiods can be easily prevented. In turn, changes in light intensity are more relevant due to seasonal changes and humans spending approximately 90% of their time indoors, which inherently reduces bright light exposure [44]. A longitudinal study found that healthy individuals in summer and wintertime did not experience a significant difference in the total time spent exposed to light, regardless of intensity [45]. However, daily exposure of environmental light that was greater than 1,000lux was far greater in summer (2.6h) when compared to winter (0.4h). Therefore, the work presented in this dissertation will focus on determining how long-term changes in daytime light intensity affect an aspect of cognitive function.

In the Nile grass rat, studies have been carried out to examine the effects of photoperiod and light intensity changes. Although grass rats exhibited depressive-like behaviors when kept in short photoperiod conditions (8h light:16h dark) in comparison to those in extended photoperiod conditions (16h light: 8h dark), animals in short photoperiods were not able to compress their general activity into the 8h light phase [46]. These results mirror activity patterns observed in humans during winter, as most individuals remain active well after sunset. However, when grass

rats were kept in an equatorial (12h light: 12h dark) dim light-dark cycle (dimLD; ~50 lux) general activity patterns were mostly similar to their bright light-dark cycle (brLD; ~1,000 lux) counterparts while still exhibiting depressive-like behaviors [47]. The lack of differences in activity patterns suggests that this chronic daytime light deficiency paradigm in which grass rats are exposed to may yield behavioral effects that are circadian-independent and would allow us to investigate the direct effects of light cognitive function properly.

Hippocampal Function and Orexin's Role in Cognition

The hippocampus (HPC) is a brain region within the limbic system that has long been linked to memory in various mammalian species that range from humans, non-human primates, and rodents [48, 49]. Even though not all types of learning and memory are dependent upon the HPC, there is a breadth of literature that confirms that several types of memory are HPC-dependent (i.e., contextual fear, episodic, and spatial). However, to say that the entire HPC is a homogenous region that is strictly involved in learning and memory would be a misconception. The dorsal (dHPC) and ventral (vHPC) subregions of the HPC have long been documented to be involved in different behavioral processes (for a review, see[50]).

Several classical studies have uncovered that there are different anatomical afferent and efferent projections for the dHPC and vHPC [51]. For example, the postrhinal cortex and the lateral band of the medial entorhinal area (MEA) send efferent projections to the dHPC preferentially while the perirhinal cortex sends and receives more projections to/from the vHPC [52]. Lesions to the vHPC in rodents appear to impact stress responses and alter emotional behavior as evidenced by lower corticosterone levels when placed in the bright compartment of a light-dark box and a lower display of anxiety-related behavior by entering the open arms of an elevated plus maze more frequently [53]. On the other hand, dHPC lesions result in deficits

regarding spatial memory within the Morris Water Maze (MWM) task while vHPC lesions did not alter the acquisition of this task [54]. This evidence strengthens the argument for the divergence of hippocampal function based on the dorso-ventral axis. However, other studies document the interconnectivity between both regions and their partial involvement in both memory and affective states [55-57]. The work presented in here will focus on studying how light modulates the dHPC for spatial navigation and examining the cellular mechanisms responsible for the behavioral outcomes, including the potential role the neuropeptide Orexin (also known as Hypocretin) may play.

Orexins are a group of neuropeptides that were discovered in the late 1990s that are mainly localized within the lateral hypothalamus (LH), the two isoforms: orexin-A (OXA) and orexin-B (OXB) bind the g-protein coupled receptors orexin 1 (OX1R) and orexin 2 (OX2R) [58]. OXA has a relative equal binding affinity to both OX1R and OX2R while OXB displays a higher binding affinity for OX2R in comparison to OX1R [59]. Because orexins and their receptors are conserved across all major vertebrate groups [60], there is a vast collection of research efforts that characterize the orexinergic system thoroughly across multiple animal models. Within the last decade, much progress has been made in dissecting the cellular mechanisms of these neuropeptides. When activated, orexin receptors generally mediate an excitatory response by increasing intracellular calcium levels [61]. Because of the wide distribution of receptors throughout the central nervous system (CNS) [62], the orexinergic system plays a significant role in various brain functions that include arousal, energy homeostasis, motor and autonomic functions, and motivated behaviors [63-66].

Because of the numerous efferent projections that the orexinergic system sends to various brain regions, orexin innervation of the HPC may play an essential role in hippocampal-

dependent learning and memory. The hippocampal formation is mainly, if not entirely, comprised of OX1Rs with sparse to no presence of OX2Rs [67, 68]. However, in diurnal grass rats, there is both abundant presence of OX1R and OX2R mRNA within the hippocampus [69] with OXA fibers being mainly present [70]. Therefore, either the OXA-OX1R or OXA-OX2R signaling pathways in the HPC may play a role in learning and memory. Of the two, investigating the OX1R pathway is more salient because there is evidence that suggests OX2R is more involved in arousal and alertness [71, 72] while OX1R seems to be involved in processing environmental stimuli [73, 74]. Additionally, most of the literature points towards the involvement of OX1Rs in hippocampal-dependent learning and memory. Administration of a selective OX1R antagonist, SB-334867, in the Cornu Ammonis 1 (CA1) and dentate gyrus (DG) regions of the dHPC results in the impairment of memory consolidation and retrieval within the MWM task [75, 76]. Moreover, it has been reported in traditional animal models that mostly express OX1Rs in the HPC that OXA, but not OXB, induces long-term potentiation (LTP) in Schaffer collateral-CA1 synapses in a kinase-dependent manner while increasing the firing rate of CA1 neurons [77, 78].

Hippocampal Synaptic Plasticity

The molecular and cellular mechanisms underlying hippocampal-dependent learning and memory have been extensively studied and well-characterized. The activity-dependent changes in the strength of synapses, formally known as “synaptic plasticity”, have been posited as a neural mechanism for the processing, storage, and representation of information crucial to memories [79]. The most recognized form of synaptic plasticity, LTP, sustains that once a synapse has been strengthened subsequent stimuli will elicit a postsynaptic response more readily [80]. One of the signature characteristics of LTP is that it exhibits lasting effects which

are dependent upon of novel protein synthesis and the remodeling of excitatory synapses. For the stabilization of strengthened synapses, increases in pre- and post-synaptic structures, as well as enhanced neuronal signaling, are the primary catalysts. Postsynaptic dendritic spines along with brain-derived neurotrophic factor (BDNF) signaling are two critical components of hippocampal synaptic plasticity that will be discussed.

Dendrites are considered to be the primary site of synaptic input derived from the axonal boutons of presynaptic neurons, although other types of synaptic interface connections exist (e.g. axo-somatic, axo-axonic, dendro-dendritic, among others) [81]. Early studies discovered that dendrites could "filter" action potentials and influence their generation within the soma due to their membranes' electrophysiological properties to offer resistance and capacitance [82, 83]. Interestingly enough, it was then found in hippocampal neurons that dendrites possessed voltage-dependent channels and could also improve the effectiveness of distal synapses through dendritic spiking [84, 85]. These classical studies laid the foundation for future research efforts in synaptic plasticity by demonstrating the electro-physical capabilities of dendrites in strengthening and dampening synapses.

In axo-dendritic synapses, synaptic input is often made onto the dendritic spine [86], which are protrusions emanating from dendritic segments. The functionality of dendritic spines within synaptic physiology is a topic that has been researched over many decades with various emerging theories over the years. In electron microscopy (EM) studies, a type 1 asymmetric synapse is classified as having an electron-dense region known as the postsynaptic density (PSD) within a dendritic spine [87, 88]. Immuno-electron microscopy (IEM) evidence uncovered that axons containing glutamate-positive boutons only contacted postsynaptic spines with a PSD, which rendered type 2 symmetric synapses as being inhibitory [89]. The presence of dendritic

spines was shown to be reliable physical markers of synapses, and the PSD was shown to contain a complex protein architecture designed to traffic and anchor glutamatergic receptors at excitatory synapses [90].

Dendritic spines fall into three morphological categories: thin, stubby, and mushroom [91]. Thin spines are characterized as having a visible neck with the head diameter not being significantly bigger. Stubby spines are described as having a wider diameter with no visible neck while mushroom spines possess a visible neck with larger head diameter. The lengthening of the PSD is directly correlated with spine expansion [92] and synaptic strength [93-95]. Thin spines have been characterized as being smaller excitatory synapses that have been newly formed while stubby ones are said to be in a transitional phase with the expansion of the PSD. However, both categories are considered to be immature spines with higher turnover rates and being more prevalent during development while becoming scarcer in the adult brain [92]. Mushroom spines have been denoted as representing the stabilized form of a synapse that persists in longer timescale (days to months) when compared to thin and spines. In the HPC, mushroom spines are likened to being "memory spines" [96]. However, recent evidence has demonstrated that hippocampal spines are dynamic and although mushroom spines in the HPC may persist for weeks and months at a time, it does not mean that they are permanent. Memory storage within the HPC is temporary due to interactions with cortical regions for permanent storage, dendritic spine populations have an average lifespan of one to two weeks and with a complete turnover within four to six weeks in both brain regions [97].

BDNF is recognized as a member of the neurotrophin family that is widely expressed across the brain, with other related molecules being Neurotrophin-3(NT-3), Neurotrophin-4(NT-4), and Nerve Growth Factor (NGF). BDNF binds two receptors: tropomyosin receptor kinase B

(TrkB) and low-affinity nerve growth factor receptor (LNGFR or p75) [98]. Originally, BDNF was thought to only play a primary role in cell differentiation and neuronal survival during development continuing throughout adulthood in mammalian species [99]. However, with the turn of the 21st century, there has been a vast amalgamation of evidence that BDNF is actively in hippocampal synaptic plasticity that is conducive to learning and memory. It has been demonstrated that BDNF is secreted pre- and post-synaptically in an activity-dependent manner to influence LTP [100].

Furthermore, it has been shown that not only does BDNF impact LTP but also plays an important role dendritic spine morphology through locally synthesized proBDNF, the precursor to mature BDNF (mBDNF), within dendritic compartments. Mutant mice lacking local proBDNF synthesis displayed impaired LTP at Schaffer collateral-CA1 synapses within the dHPC along with a reduced head diameter of dendritic spines [101]. A goal of this dissertation is to determine if light-modulated orexinergic signaling influences hippocampal synaptic plasticity through similar cellular mechanisms that have been previously described.

Lighting Paradigm and Orexin

Within the laboratory of Dr. Lily Yan at Michigan State University, we have established a chronic daytime light deficiency paradigm that has resulted in the alteration of several behavioral responses in diurnal animals that we believe are because of light's NIF effects [47, 102]. Interestingly, we have also observed that OXA expression is significantly attenuated when our animal model is exposed to chronic dim lighting conditions [103]. The work presented in this dissertation will examine: (1) if chronic daytime light deficiency impacts hippocampal-dependent memory in diurnal grass rats; (2) how chronic dim lighting can affect female grass

rats and determine any differences from their male counterparts; and (3) dissect the role of the OXA-OX1R in light's modulation of hippocampal function.

Summary of Dissertation Experiments

Chapter 2: Here I exposed male grass rats to four weeks of either chronic dim (dimLD) or bright (brLD) lighting conditions. Afterwards, I trained and tested them in the MWM task to assess their spatial learning and memory, following behavioral testing I examined hippocampal BDNF immunoreactivity as a marker for synaptic plasticity. In a separate cohort of animals, I first exposed them to four weeks of chronic dim lighting conditions followed by an additional four of bright lighting conditions ("bright light therapy") to assess if the effects of chronic dim light were reversible. Additional animals were utilized to assess the potential impact of chronic daytime light deficiency on general hippocampal morphology via Golgi staining. More detailed morphological assessments were carried to determine which hippocampal dendritic subtypes were most affected by chronic dim lighting conditions by introducing a viral vector via stereotactic surgery for visualization and reconstruction of dendritic processes.

Chapter 3: In this chapter, I subjected female grass rats to the same conditions as described in Chapter 2 with the exception that no "bright light therapy" was conducted. Hippocampal BDNF was assessed both by immunohistochemistry and Western Blotting. Phosphorylation of BDNF's receptor, TrkB, was assessed via Western Blot to examine further light's potential impact on BDNF signaling in female grass rats. Detailed morphological assessments were carried out with the introduction of viral vectors, as mentioned in Chapter 2.

Chapter 4: The experiments presented in this chapter will try to uncover the role of the orexinergic system in light's modulation of hippocampal-dependent learning and memory. The first half of this chapter I will attempt to determine if intranasal (IN) administration of OXA can

prevent HPC-dependent learning and memory deficits brought on by chronic daytime light deficiency. If valid, I will also attempt to examine at which learning phase does IN-OXA exert its effect. Additionally, I will also look at the rate of phosphorylation of CaMKIIα and GluR1 via Western Blot as indicators of hippocampal LTP induction. In the second half of this chapter, I will introduce a viral vector designed to knockdown OX1Rs within the dHPC and determine if the disruption of the OXA-OX1R signaling pathway from the LH to the dHPC produces a similar behavioral phenotype that is caused by chronic daytime light deficiency. Detailed morphological analyses will be conducted similarly as mentioned in the previous chapters. Hippocampal BDNF expression will be assessed in both halves of this chapter via Western Blot.

CHAPTER 2:

Light modulates hippocampal function and spatial learning in a diurnal rodent species: A study using male nile grass rat (*Arvicanthis niloticus*)

The work presented in this chapter has been published in manuscript form.
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Introduction

Environmental lighting conditions influence a vast array of physiological and behavioral processes in humans, i.e., circadian rhythms, alertness/ arousal, as well as mood and cognition [3, 104, 105]. The effects of light in regulating cognitive processes have been documented across diverse populations, with brighter illumination yielding improved cognitive performance. For example, brighter illumination in the classroom enhances the performance of elementary school students in math and reading [25, 26, 106, 107]; bright office lighting improves the performance of adults in the work environment [27, 108, 109] and bright light therapy has been shown to attenuate cognitive deterioration in mild/early-stage dementia [28-30]. However, the neural mechanisms through which light modulates cognitive functions are not well understood.

For diurnal species, including humans, light promotes alertness, which is essential for optimal cognitive function [1]. Humans receiving bright light exposure during the day have lower sleepiness and fatigue scores compared to those in a dim light condition [110]. Neuroimaging studies have shown that daytime bright light exposure instantly increases activity in the subcortical regions that support alertness/ arousal even before affecting cortical areas involved in cognitive processes and performance [111]. Similar results are obtained when using blue-enriched light at a ~460nm wavelength [7, 112], which is the preferred wavelength for the retinal ganglion cells that are responsible for nonimage-forming photoreception [7, 112-114].

Light also modulates human attention and executive functions involved in cognitive processing. Measuring brain activities using electroencephalogram (EEG), shows that daytime exposure to blue light increases the amount of attentional resource allocated to cognitive tasks [115, 116]. Furthermore, bright light therapy has been used in patients with attention-

deficit/hyperactivity disorder, who show improvement in measures of both attention and executive function [117].

In addition to the acute effects of bright-light exposure on arousal and attention, chronic changes in ambient lighting conditions can produce long-lasting effects on brain and behavior. For example, laboratory rats housed in constant light during early development are resistant to the disruptive effects of constant light on circadian rhythms throughout their adulthood, suggesting that alterations in ambient illumination can lead to long-term changes in the brain [118]. Mice housed under different photoperiods or day-length over early development also show enduring difference in their dorsal raphe serotonin neurons, including their electrical properties and neurotransmitter content [119]. In postmortem human brain tissue, the number of midbrain dopaminergic neurons is higher in those who died in summer compared to those in winter [120]. Our own work using Nile grass rats (*Arvicanthis niloticus*), a diurnal rodent species, shows an increased number of dopaminergic and serotoninergic neurons in animals that had been housed over 4 weeks under daytime bright light (~1000 lux) as compared to those kept under daytime dim light (~50 lux) [47, 121]. On the basis of these findings, we hypothesized that long-lasting changes in the brain, beyond temporary enhancement of arousal or attention, are likely to contribute to the superior cognitive performance associated with brighter illumination.

To test this hypothesis, the present study utilized the diurnal Nile grass rat and hippocampal-dependent spatial learning/memory as model systems to explore the neural mechanisms through which ambient lighting conditions impact cognitive functions. Spatial learning and memory was assessed using the Morris Water Maze (MWM) task, which has been has been widely used in rodent species [122, 123]. Successful performance in MWM task has been shown to rely upon an intact hippocampus [124-126] and is strongly correlated with

hippocampal expression of brain-derived neurotrophic factor (BDNF) and dendritic plasticity [127, 128]. Therefore, we focused our investigation on the hippocampus by examining its expression of BDNF and its dendritic spine morphology. The present study provides novel insights into the mechanisms responsible for the effects of ambient light on cognitive function, and has identified the grass rat as a useful diurnal animal model to further elucidate the underlying neural substrates for the behavioral effects of differential light exposure.

Experimental Procedures

Subjects

Male unstriped Nile grass rats (*Arvicanthis niloticus*) from our breeding colony at Michigan State University were used for all experiments. All animals were entrained to a 12:12 h light-dark (LD, ~300 lux during the day) cycle and were given food (PMI Nutrition Prolab RMH 2000, Brentwood, MO) and water ad libitum. All grass rats were group-housed prior to the start of the behavioral testing and then single-housed for the duration of the study in Plexiglas cages (34 3 28 3 17 cm3), under either a 12:12 h bright light-dark (brLD, ~1,000 lux during the day) or dim light-dark (dimLD, ~50 Lux) cycle as described in our previous studies [47, 103]. A PVC tube was provided in the cage as a form of enrichment and as a hut for the animals. All experiments were performed in compliance with guidelines established by the Michigan State University Institutional Animal Care and Use Committee (IACUC), and the National Institutes of Health (NIH) guide for the Care and Use of Laboratory Animals.

Morris Water Maze

Three cohorts of animals (n= 8/lighting condition) were used in this experiment. In the first cohorts, animals were housed in either brLD or dimLD for 1 week prior to being trained on the Morris Water Maze (MWM); while in the 2nd cohort, animals were housed in each condition

for 4 weeks prior to MWM training. For the 3rd cohort, animals either remained in the colony condition (~300 lux) and then transferred to dimLD for 4 weeks prior to training on the MWM, or housed in dimLD for 4 weeks before being transferred to brLD for an additional 4 weeks prior to training. For all cohorts and conditions, during the last week before training, the animals were handled daily for 10 min to reduce novelty-induced stress that may stem from the experimenter's handling of the animals [129]. Handling was performed in the animals' home cage in the behavioral testing room. Animals were trained and tested during *zeitgeber* time (ZT) 5–7, lights on was defined as ZT0; the light intensity in the testing room was ~300 lux. Training on the MWM was performed as previously described using a circular pool (60 cm depth x 122 cm diameter) with a platform (15 cm diameter) located 2 cm under the water level and ~30 cm away from the perimeter of the pool [130]. The water was made opaque with nontoxic white tempera paint and kept at 26±2°C; different geometrical cues were posted up on each wall of the room for spatial orientation. Prior to the hidden-platform training, animals underwent a one-day cued-platform training, during which the water was clear and the platform was kept above water. This was done to ensure that any deficits seen during the hidden platform training were not due to impaired motor functions [123]. As a prerequisite, all animals included in the following experiments located the platform in less than 2 min when it was visible. For the hidden platform procedure, each animal completed two training trials per day over 5 days with each trial being a maximum of 2 min in length with an inter-trial interval of 30 s. If the animal failed to locate the platform at the end of the 2 min period, it was guided towards the platform, and given a latency score of 120 s. On the sixth day, reference memory was tested 24 h after the last training session by removing the platform from the MWM and allowing each grass rat to swim for 1 min to measure the following parameters: time spent in the goal quadrant where the platform had been

located, swim speed, and thigmotaxis, i.e., time spent swimming next to the wall [131]. All behavior videos were loaded into Noldus *Ethovision* (*XT* 8.5, Noldus Information Technology, Netherlands) and scored by an experimenter who was blind to the experimental conditions.

Immunohistochemistry (IHC)

Animals tested in the MWM were left undisturbed for two days before being used for the IHC analysis. Another group of animals that was housed under the same lighting conditions, i.e., 4 weeks of either brLD or dim LD, but without behavioral training/testing, was also used for the IHC analysis. All animals were transcardially perfused at ZT 5–7 with saline followed by 4% paraformaldehyde (PFA). Brains were post-fixed and cryo-protected, then three alternate sets of 40 mm sections were collected using a cryostat. Ten sequential sections containing the dorsal HPC from one alternate set were processed for IHC using anti- BDNF primary antibody (1:5,000, raised in rabbit, ab101747, Abcam, Cambridge, UK). The specificity of the antibody in grass rats has been verified in a previous study [130]. The IHC procedures were carried out as described in our previous studies using 3,3'-Diaminobenzidine (DAB) and 4% Nickel Sulfate for colorimetric reaction [46, 47, 103, 132]. After the IHC reaction, sections were mounted, dehydrated/clarified and then cover-slipped using Permount (Fisher Scientific, NH). Photomicrographs of the dorsal hippocampus were taken using a CCD camera attached to a Nikon light microscope and analyzed using Image J (NIH) as described in previous studies [46, 47, 103, 132]. The number of BDNF-ir cells was determined for the CA1, CA3, and dentate gyrus (DG) subregions of the hippocampus with a 200 x 400 mm counting box (Figure S1.1, See Appendix A).

Golgi Staining

Behaviorally naïve animals were used in this study. Grass rats were housed in either brLD or dimLD (n= 7/condition) for 4 weeks prior to transcardial perfusion (at ZT 5–7) with a phosphate buffer followed with a Rapid-Golgi fixative solution [modified from [133]]. Brains were post-fixed in the same solution for 24 h, then transferred to 3% potassium dichromate for three days before immersion in 1% $AgNO_3$ for eight days. Brains were placed in 20% sucrose for 48 h prior to sectioning at 100 mm using a cryostat. Sections were processed through an ethanol dehydration series and were clarified with xylene. Sections were mounted onto gelatin-coated slides and cover-slipped with Permount (Fisher Scientific, NJ). For quantification, images of dendritic spines were captured using a CCD video camera (CX9000, MBF bio- science, VM, USA) attached to a light microscope using an oil immersion lens (Nikon Instruments, NY) and spines were quantified using ImageJ with the AnalyzeSkeleton plug-in (Ignacio Arganda-Carreras, http://fiji. sc/wiki/index.php/). CA1 apical dendritic spines were analyzed from 20mm segments of four distinct dendritic branches per neuron, a total of six neurons were analyzed per brain [134].

Spine Morphology

Animals were housed in either brLD or dimLD for 4 weeks (n= 6/condition) without behavioral training or testing (behaviorally naïve). They then received bilateral injection into dorsal hippocampus of herpes simplex virus expressing green fluorescent protein (HSV-GFP, Massachusetts Institute of Technology Viral Core Facility) [135]. Needles of 26-gauge (Hamilton Com- pany, Reno, NV) were placed bilaterally at the following coordinates from bregma: 20.1 mm A-P (anteroposterior); 6 2.0 mm L-M (mediolateral); 22.7 mm D-V

(dorsoventral) from brain surface. Purified high-titer HSV-GFP (0.5 μL) was infused at a rate of 0.1 μL/min, after infusions the needle rested at the site for 5 min prior to extraction.

After 48 h post-surgery to allow for maximal GFP expression, animals were perfused transcardially with PBS followed by 4% paraformaldehyde. Sections were obtained at 100 μm thickness and mounted onto subbed glass slides with ProLong® Diamond Antifade Mountant (ThermoFisher Scientific, Waltham, MA). For detailed morphological analyses of dendritic spines, samples were imaged on a Nikon A1Rsi laser scanning confocal microscope utilizing a 100x Plan Apo TIRF DIC-oil immersion objective (total magnification of 1,000x). To visualize GFP, the samples were excited with a 488nm laser and the fluorophore emission was captured by a 525/50 band-pass (BP) filter. A z-stack was obtained for each sample for dendritic spine analysis.

For each animal, five neurons (two dendritic segments/neuron) were analyzed. Z-stacks were used to achieve three-dimensional reconstruction utilizing the NeuronStudio freeware morphometric program, which allows for accurate visualization and aids in reducing experimenter bias [135]. Dendritic spine density for the dendritic segments was quantified and grouped by subtypes (e.g., thin, stubby, and mushroom) based on neck length and head diameter. Thin and mushroom spine subtypes are classified as having visible necks with the major difference being that the head diameter of thin spines is not notably different from the neck diameter, while mushroom spines' head diameters are clearly larger than their neck diameter. Stubby spines are characterized as having a large head diameter along with no neck presence [96, 136].

Data Analysis

Statistical analysis was performed using SPSS (version 24, IBM, Armonk, North Castle, NY). For MWM behavioral data, the latency to reach the platform was analyzed using 2 x 5 Mixed ANOVAs with lighting condition as the between–subjects factor and training days as the repeated measures factor for trials 1 and 2 separately. In the case that there was a significant interaction, Holm-method comparisons were used to evaluate group differences across all five training days; when there was no interaction, only main effects were interpreted. Two-tailed independent samples student's t-tests were used to assess group differences on the amount of time spent in the goal quadrant, swim speed, and thigmotaxis (i.e., time spent swimming in the periphery not representative of a search pattern) during the probe tests. The number of BDNF-ir cells in each sub-region (Figure S1.1, See Appendix A) and dendritic spine density in CA1 were compared between lighting conditions using two-tailed independent samples student's *t*-tests. The threshold for statistical significance for all analyses was established at $p < 0.05$.

Results

Chronic daylight deficiency impairs MWM performance

For the first trial of each day, both groups kept under either dim- or brLD for 4 weeks showed significant improvement in their performance (i.e., latency to find the platform) over the 5 training days, but the performance of the brLD group was superior to that of the dimLD animals (Figure 1.1a; main effect of training days: $F(4,56)= 16.493$, $p < 0.001$; main effect of lighting condition: $F(1,14)= 4.652$, $p < 0.05$). There was no significant interaction between training days and lighting condition ($F(4,56)= 0.953$, $p > 0.05$). That group difference was absent for trial 2 ($F(1,14)= 1.377$, $p > 0.05$), which was conducted 30 s after trial 1 (Figure 1.1b). By the last two training days, the majority of the animals successfully located the platform during

training trails (Figure S1.2, See Appendix A). During the probe trial when the platform was removed, the brLD animals concentrated the search within the goal quadrant, in contrast to the dimLD animals (Figure 1.1c). Comparison of the time spent on the goal quadrant by each group showed a significant difference with the brLD spending more time on the quadrant than the dimLD group (Figure 1.1d; $t(14)= 2.98$, $p= 0.01$). The performance of the dimLD group was not significantly different from chance (15 s; $t(7)= 20.057$, $p= 0.956$). The groups did not differ significantly with respect to swim speed ($t(14)= 0.002$, $p > 0.05$) and thigmotaxic behavior ($t(14)= 20.76$, $p>0.05$). In contrast to the animals housed in each condition for 4 weeks, identical testing of animals kept in brLD or dimLD for just one week did not result in group differences on any of the dependent variables (Figure S1.3, See Appendix A).

Impaired MWM performance resulting from daytime light deficiency can be restored by transferring to brLD condition

To determine if the impairments in spatial learning/memory due to light restriction are reversible, the animals initially housed for 4 weeks in dimLD were transferred to brLD and kept there for 4 weeks before testing. The transferred animals showed superior performance as compared to those kept in dimLD. For trial 1 there was a significant main effect of training days ($F(4,56)= 15.05$, $p < 0.001$) and housing condition on the latency to reach the platform ($F(1,14)= 12.942$, $p= 0.003$), with no significant interaction (Figure 1.2a; $F(4,56)= 2.38$, $p= 0.062$).

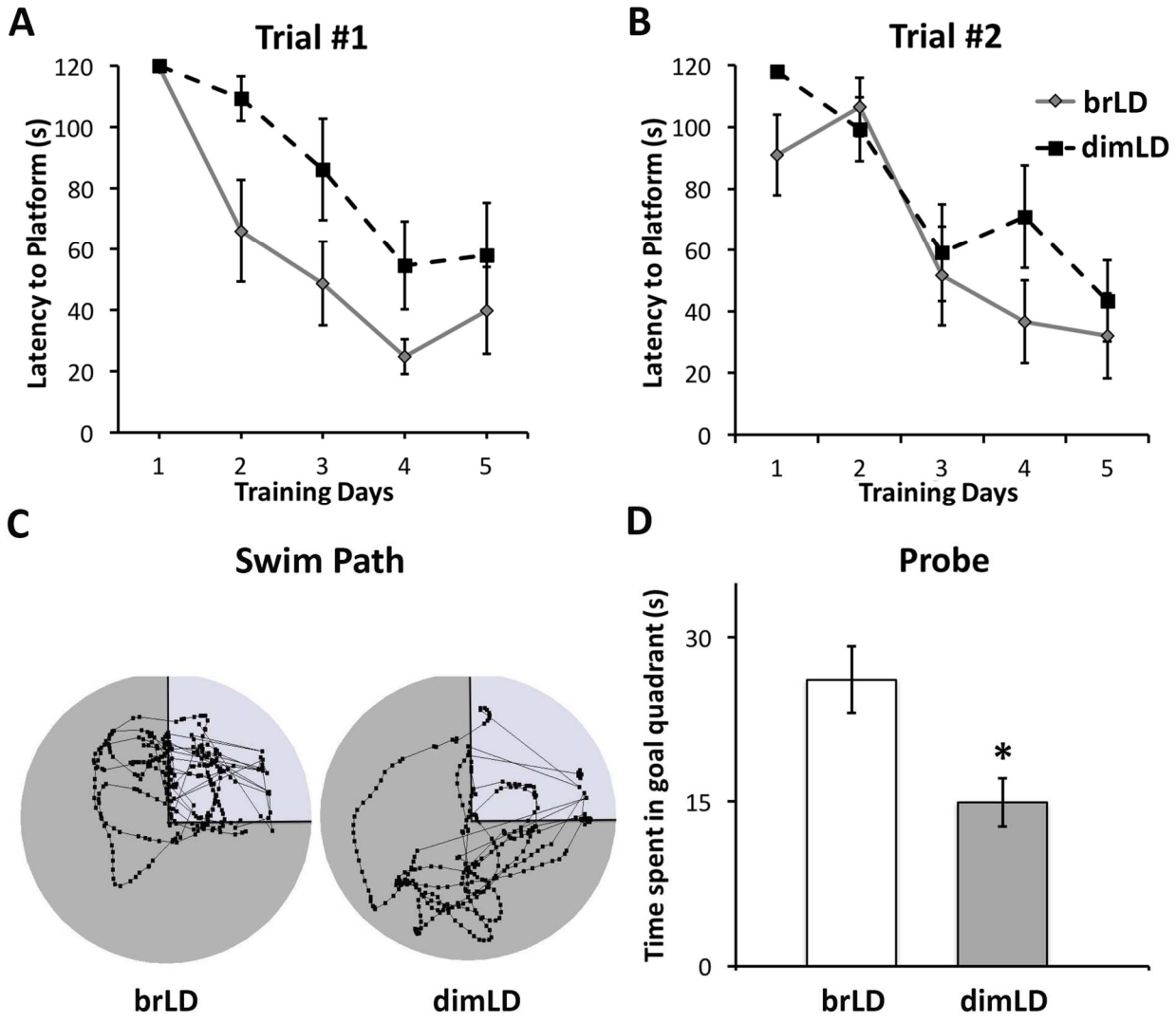

Figure 1.1 Impaired MWM performance of grass rats housed in dimLD as compared to those in brLD condition over 4 weeks. (a) Latency of animals to locate the platform during trial 1 (24 h delay) over the 5 training days. Grass rats housed in brLD were able to locate the platform significantly faster in the than those housed in dimLD (main effect of training days: $F(4,56)= 16.493$, $p < 0.001$; main effect of lighting condition: $F(1,14)= 4.652$, $p< 0.05$); interaction between training days and lighting condition ($F(4,56)= 0.953$, $p > 0.05$). (b) Latency of animals to locate the platform during trial 2 (30 s delay), there were no significant differences between the two groups. (c) Representative track plots of a grass rat in each lighting condition during the probe trial (with goal quadrant highlighted). (d). Grass rats housed in brLD nearly spent twice as much amount of time searching for the platform in the goal quadrant in the probe test when compared to grass rats in the dimLD group. *, $p < 0.05$

Individual group comparisons showed superior performance for the animals in the reversal condition (i.e., dimLD-brLD) over those in the dimLD group for days 2, 4, and 5 of training. No group differences were detected for the latency data for trial 2 (Figure 1.2b). During the probe trial, animals transferred from dimLD to brLD concentrated the search for the platform within the goal quadrant in contrast to the dimLD animals (Figure 1.2c). The transferred animals also spent more time in the goal quadrant than the dimLD group (Figure 1.2d, $t(14)= 4.387$, $p= 0.001$). There were no significant differences in swim speed ($t(14)= 0.488$, $p > 0.05$) or thigmotaxis ($t(14)= 0.116$, $p > 0.05$) between groups.

Ambient lighting condition modulates hippocampal BDNF expression

BDNF-ir in the hippocampus was reduced in the dimLD group when compared to the brLD group (Figure 1.3a). The average number of BDNF- ir cells was analyzed in CA1, CA3, and DG. The number of BDNF-ir cells were consistently lower across the three areas for the dimLD group, although statistical significance was reached only for CA1 ($t(10)= 3.05$, $p= 0.012$, Figure 1.3b). The data reported here are from animals that had been through MWM training. A separate cohort of animals without any behavioral testing was also compared for BDNF-ir (Figure S1.4, See Appendix A). Similar results were obtained, with lower number of BDNF-ir cells in the CA1 of dimLD as compared to brLD condition in these naïve animals ($t(10)= 6.798$, $p < 0.001$). Similar to the behavioral reversal in MWM performance seen when the dimLD animals were transferred to brLD for 4 weeks, there was an increase in hippocampal BDNF-ir in the transferred animals as compared to those kept in dimLD (Figure 1.3c). A significant increase was observed in both CA1 ($t(10)= 7.307$, $p < 0.001$) and CA3($t(10)= 4.183$, $p= 0.002$).

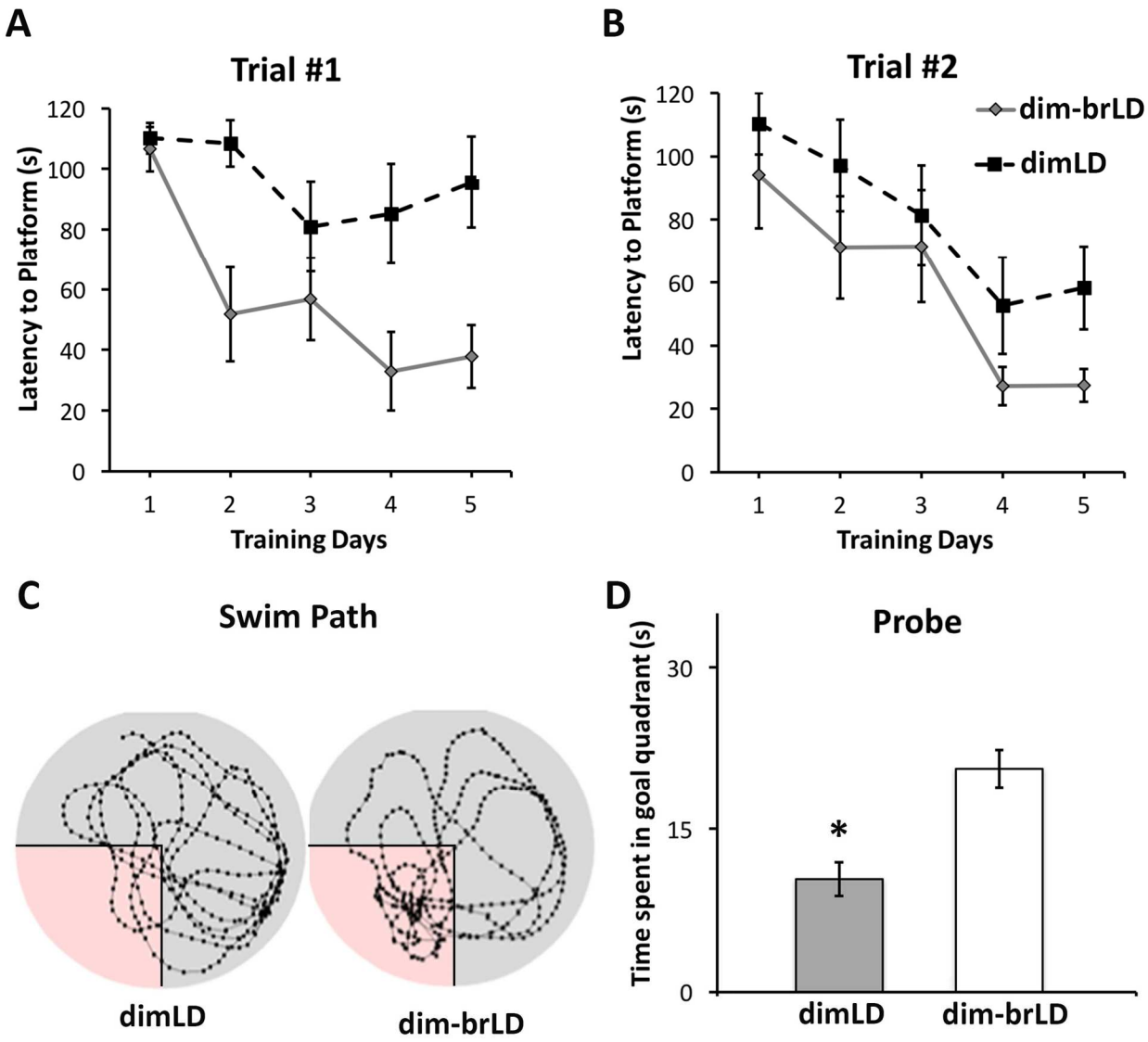

Figure 1.2 Subsequent brLD housing (dim-brLD) restored the impaired MWM performance of animals housed in dimLD conditions for 4 weeks. (a) Latency of animals to locate the platform during the first trial (main effect of training days: ($F(4,56)= 15.05$, $p < 0.001$); main effect of housing condition: ($F(1,14)= 12.942$, $p= 0.003$); interaction between training days and housing condition: $F(4,56)= 2.38$, $p= 0.062$). (b) Latency of animals to locate the platform during the 2nd trial, there were no significant differences between the two groups. (c) Representative track plots of a grass rat in each lighting condition during the probe trial (with goal quadrant highlighted). (d). Time spent searching for the platform in the goal quadrant in the probe test. *, $p < 0.05$

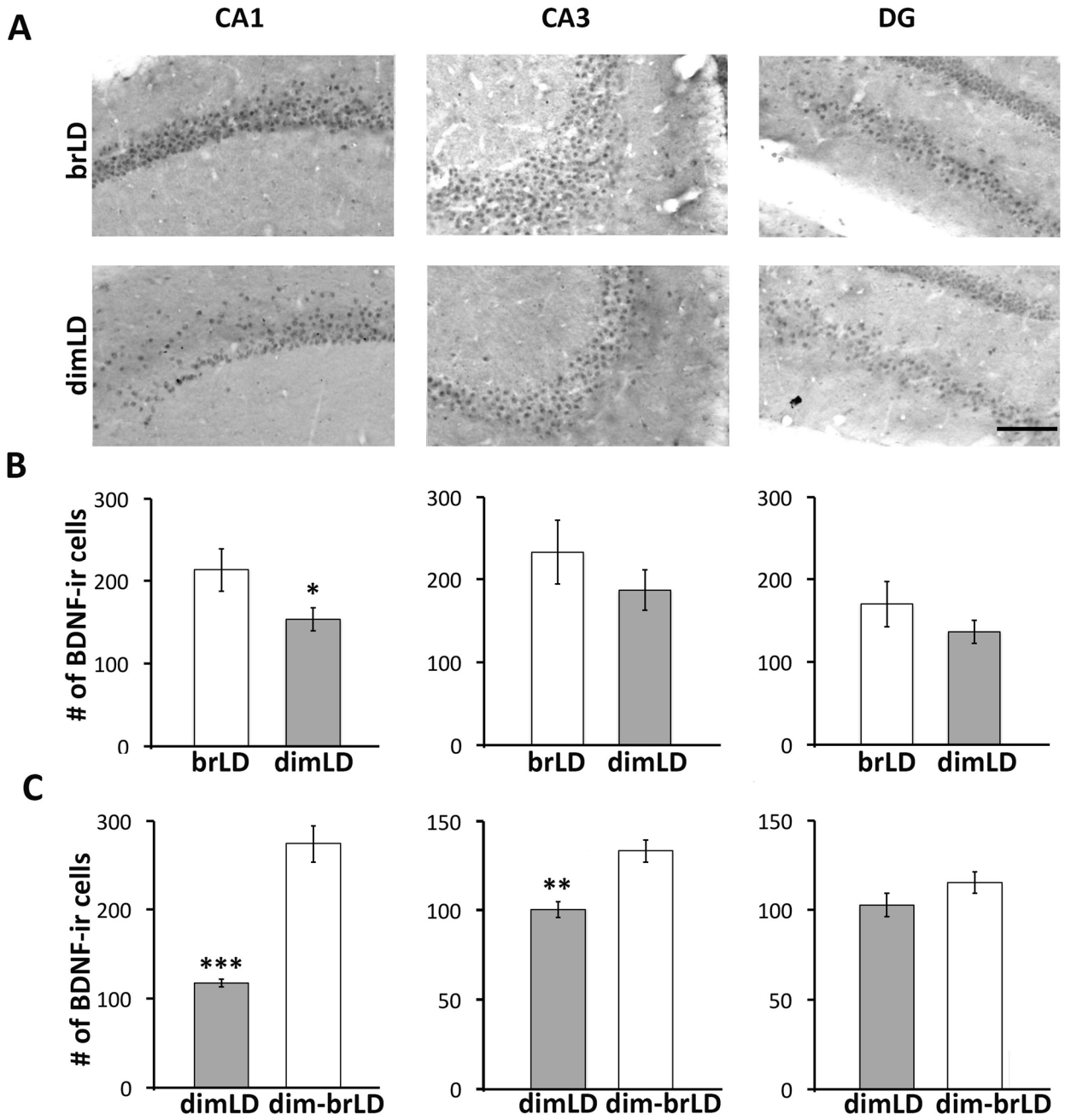

Figure 1.3 Ambient light condition modulates hippocampal BDNF expression. (a) Representative photomicrographs of BDNF immunochemical staining within the CA1, CA3, and DG of the hippocampus of grass rats housed in brLD or dimLD condition. (b) Number of BDNF-labeled cell bodies in each subregion of the hippocampus in animals housed in brLD or dimLD condition. (c) Number of BDNF-labeled cell bodies in each subregion of the hippocampus in animals housed in dimLD and those initially housed in dimLD then switched to brLD. Scale bar, 100 μm. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Ambient light modulates CA1 apical dendritic morphology

In addition to BDNF expression, ambient light also modulates structural plasticity in the hippocampus. The morphology of golgi-stained apical dendrites was analyzed in CA1 of animals from different lighting condition (Figure 1.4). When the brLD and dimLD groups were compared (Figure 1.4a,b), there was a significant reduction of apical dendritic spine density in the dimLD ($t(8)= 5.103$, $p= 0.001$). Following transferring to the brLD condition (Figure 1.4c,d), the dimLD-brLD group showed a significant increase in apical dendritic spine density as compared to those kept in dimLD ($t(10)= 10.062$, $p < 0.001$).

The morphology of apical dendritic spines was further analyzed using HSV-GFP expression in hippocampal neurons (Figure 1.5). Examples of labelled apical dendrites from animals kept four weeks in brLD or dimLD are shown in Figure 1.5a. Group comparisons of the abundance of different types of spines in CA1 found significant higher density of mushroom ($t(9)= 2.680$, $p<0.05$) and stubby spines ($t(9)= 4.605$, $p= 0.001$) in the BLD group compared to the DLD animals, with no significant group differences for density of thin spines (Figure 1.5b).

Discussion

We show here that in diurnal Nile grass rats, chronic conditions of ambient lighting can influence cognition in a way similar to that observed in humans, such that bright light is beneficial over dim light for cognitive performance. In addition, we found that lighting condition can modulate the level of hippocampal BDNF expression as well as structural plasticity within the hippocampus.

The Nile grass rat is a well-established diurnal rodent model that has been used in various research areas including circadian rhythms and sleep/arousal systems to show how the control of those functions differs from that of nocturnal rodents [70, 137-142]. Because of the different, and

often opposite, effects of light on diurnal and nocturnal species (e.g., light promotes wakefulness/arousal in diurnal animals including humans, but induces sleep in nocturnal ones), a diurnal model is of crucial importance for a mechanistic understanding how light modulates cognition in humans [31, 32].

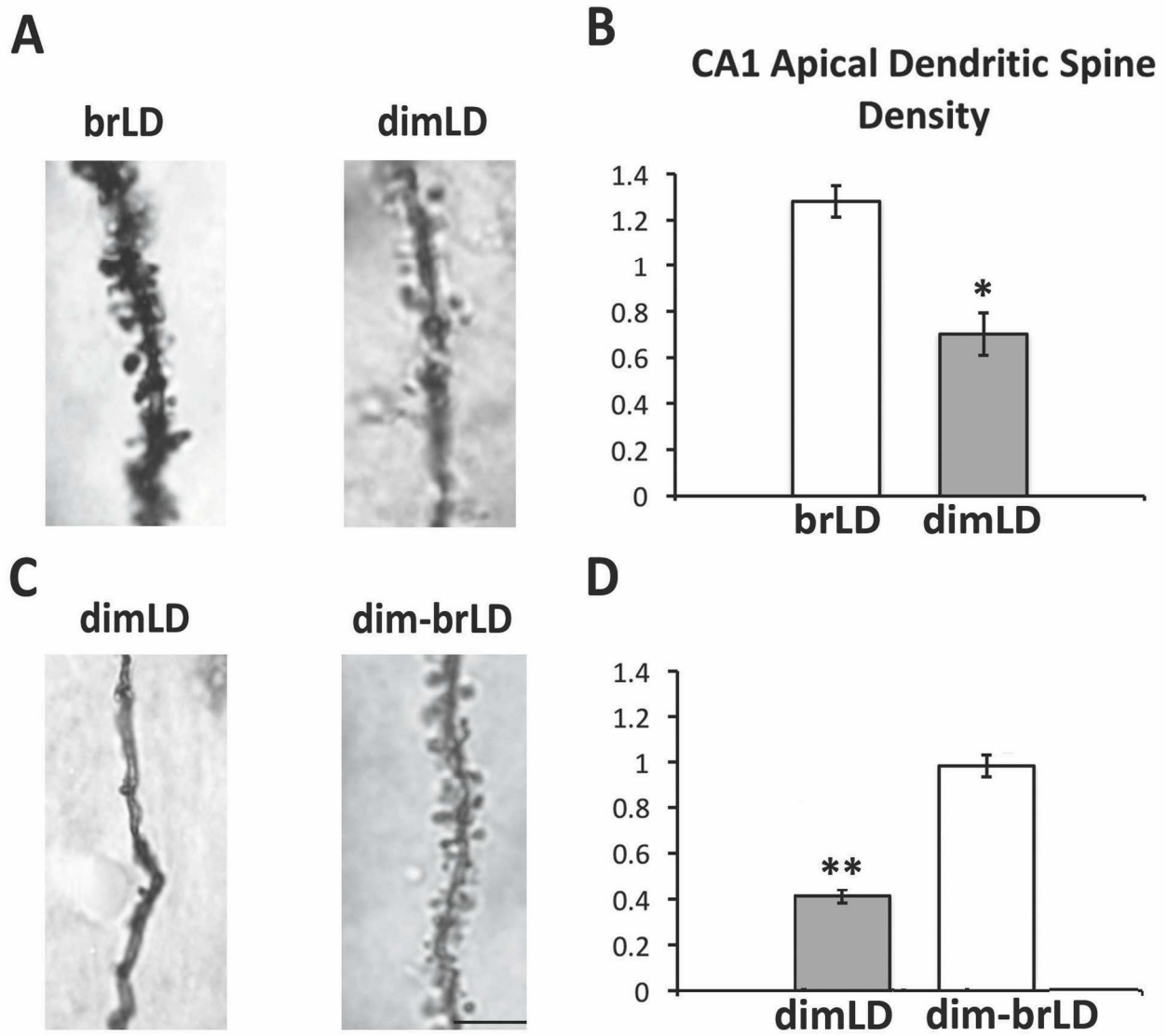

Figure 1.4 Golgi staining of CA1 apical dendrites. (a) Representative photomicrograph and (b) quantification of dendritic spines of grass rats housed in either brLD or dimLD condition for 4 weeks. (c) Representative photomicrograph and (d) quantification of dendritic spines of grass rats housed in dimLD or initially in dimLD then transferred to brLD. Scale bar, 5 μm. *, $p < 0.01$; **, $p < 0.001$

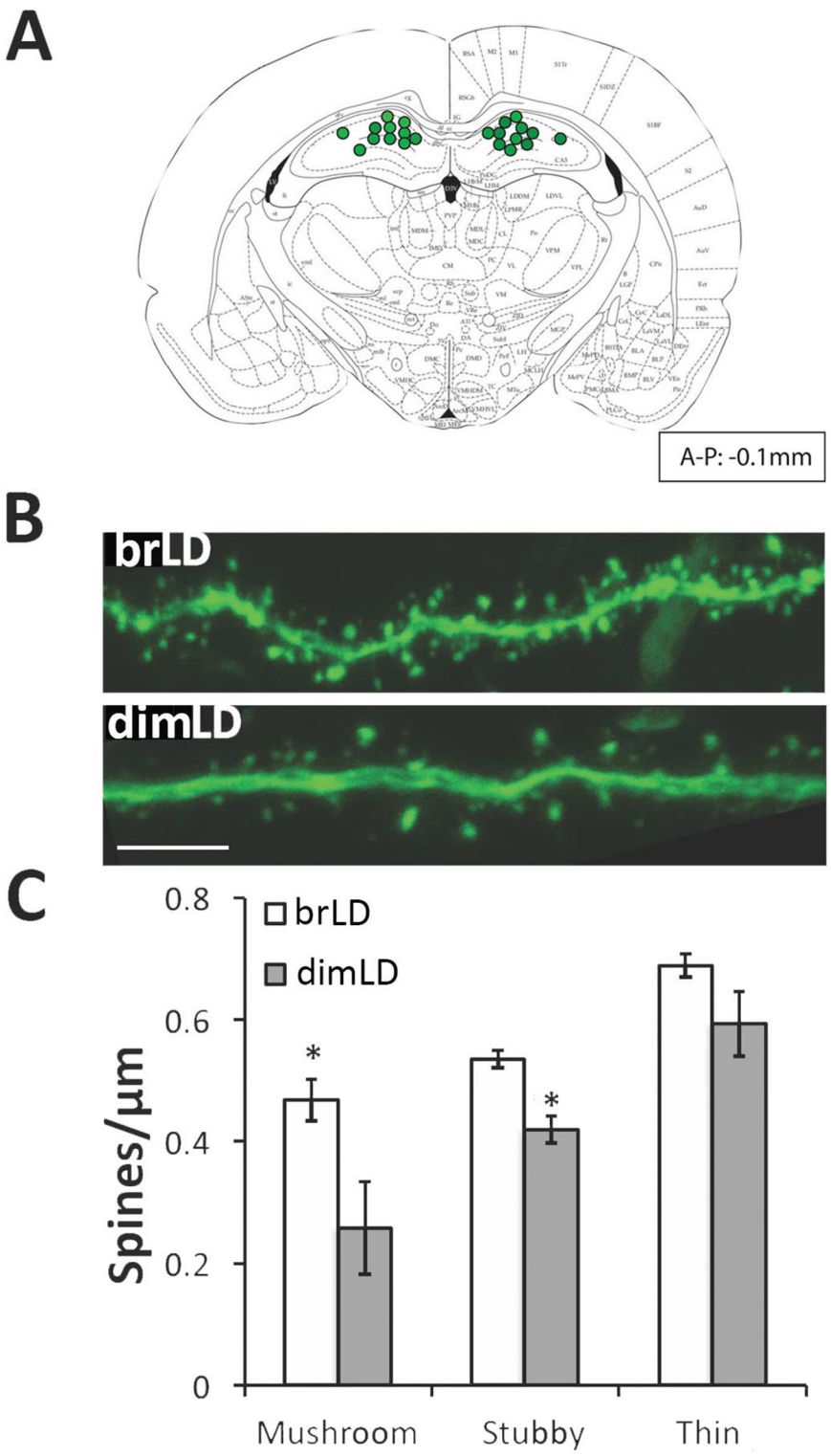

Figure 1.5 Hippocampal apical dendrites visualized by HSV-GFP expression. (a) The injection sites of the HSV-GFP. (b) Representative photomicrophs of HSV-GFP expression. (c) Quantification of the density of dendritic spine sub-types. Scale bar, 5 µm. *, $p < 0.01$

Diurnal rodent models i.e., Mongolian gerbils, fat sand rats and Nile grass rats have been used for investigating the impact of lighting condition on brain and behavior in various lighting paradigms including total darkness, short-photoperiod or dim light exposure at night [143-145]. The lighting paradigm used in the present study was designed to manipulate daytime light intensity while keeping the day-length or photoperiods constant. By having photoperiods remain constant, our findings aim to be more ecologically relevant to humans because, unlike animals in nature under sunlight, much of our living environment is comprised of artificial light. Therefore, we do not experience drastic changes in day-length, but rather variations in the quality of light i.e., spectrum or intensity [45].

After being kept in dim light during the day (dimLD) for 4 weeks, grass rats showed a deficit in the MWM task compared to the perform- ance of animals kept under brLD for the same duration. The deficit was evident for both the first trial of each training day and for the probe test, in which the amount of time the dimLD animals spent in the goal quadrant was at chance level (Figure 1.1). There were no group differences for animals kept in the two lighting conditions for just one week, thus suggesting that the detrimental effects of dim light develop over time (Figure S1.3, See Appendix A). Further, since both groups were tested under identical intermediate lighting conditions, the superior performance of the animals in brLD for 4 weeks is not due to the acute effects of bright light on performance, as has been reported in human studies [30, 146, 147]. Swimming speed did not differ across groups; therefore, group differences are not likely to reflect deficits in motivation [148] or sensory-motor functions [123]. Even though, dimLD housing has been shown to be anxiogenic for grass rats in the open field test [102], the lack of group differences in the display of thigmotaxis in the MWM suggests that the memory deficit of the dimLD animals is unlikely the result of enhanced anxiety during training/testing.

When the dimLD grass rats were rehoused under the brLD condition for another 4 weeks, the ani- mals performed significantly better than those in dimLD, suggesting that subsequent exposure to bright light can restore impaired spatial memory due to previous light restriction (Figure 1.2).

Interestingly, the effect of long-term light restriction on latency to find the platform during training, was only significant for trial 1 of each day (Figures 1.1a and 1.2b), suggesting that the retention of the memory for the platform location was impaired in the dimLD animals after a 24 h interval but not for the 30 s between-trial interval; a similar conclusion is supported by the results of the probe trial, which occurs a day after the last training trial. However, the effect of lighting condition on latency to find the platform was not significant for trial 2 of each day (Figure 1.1b and 1.2b), indicating that exposure to the water maze on trial 1 of each day was sufficient to bring the performance of the dimLD animals to the level of the brLD group in the second trial 30 s later. In the MWM, working memory, which involves the prefrontal cortex [149], is engaged as the animal searches for the escape platform on subsequent trials of the same training day, whereas hippocampal-dependent reference memory is necessary for remembering the location of the platform from one training day to the next. Thus, the normal performance of dimLD animals on trial 2 may reflect an intact working memory and lack of dysfunction in the prefrontal cortex. Alternatively, the experience of trial 1 each day may reactivate a relatively weak reference memory [150], which then supports the normal performance of the dimLD animals on trial 2. Regardless of the possible explanations for the improved performance of the dimLD animals on trial 2, our results point to an inability to consolidate a robust hippocampal-dependent memory over a 24 h interval. The results collectively suggest that long-term (4 weeks) light deficiency impairs consolidation of spatial memories, which is indicated by the rapid

forgetting over a 24 h interval displayed by the dimLD animals. This rapid forgetting of reference memory in the MWM has been seen in studies with other animal models of hippocampal deficits e.g., epilepsy [151], and hippocampal insulin resistance [152].

MWM performance has been linked to hippocampal expression of BDNF [128], a member of the neurotrophin family of growth factors, which has been shown to be involved in learning and is crucial for long-term memory [100, 153, 154]. Analyses of hippocampal BDNF expression and apical dendritic spines revealed a significant effect of ambient light on the structural plasticity of the hippocampus. Following 4 weeks of dimLD housing, there was a significant reduction in the number of BDNF-ir cells in the CA1 sub-region, compared to the animals in brLD and to those initially housed in dimLD then switched to brLD for another 4 weeks (Figure 1.3). It is noteworthy that the brain samples from the two cohorts of animals i.e., 4 weeks in brLD versus dimLD (Figure 1.3b) and 4 weeks in colony lighting (~300 lux) followed by 4 weeks dimLD versus 4 weeks dimLD followed by 4 weeks brLD (dim- brLD, Figure 1.3c) were processed for ICC separately, therefore, the results cannot be directly compared. Nonetheless, the differences between the dimLD and dim-brLD in the last cohort (Figure 1.3c) appear to be greater than that in Figure 1.3b when dimLD and brLD were compared. The greater differences in the last cohort (Figure 1.3c) may due to the interaction between the lighting condition and prolonged (8 weeks) singly housing of the animals. Since these animals had been through MWM training/testing, the differences observed in BDNF-ir could have resulted from their housing condition, or alternatively, from their experience with the MWM, or the interaction of the two factors. Thus, we repeated the analysis in naïve animals. The results revealed a similar pattern with higher BDNF-ir in brLD as seen in the animals exposed to MWM training, thus suggesting that the difference in BDNF-ir was indeed due to the effects of the lighting condition

and not the result of differential performance of the two groups on the MWM (Figure S1.4, See Appendix A).

BDNF signaling modulates dendritic spine growth in the CA1 [155]. The growth of dendritic spines in the hippocampus, particularly within the CA1 region, has been linked to the formation of new synapses and improved learning and memory [94, 156, 157]. We found reduced CA1 apical dendritic spine density in dimLD animals compared to brLD and dimLD-to-brLD groups (Figure 1.4), suggesting a possible change in CA3-CA1 connectivity, a crucial circuit for spatial memory [158, 159]. Following 4 weeks of rehousing the dimLD group in the brLD condition, we found a significant increase in both BDNF-ir and dendritic spine density in the hippocampus. These findings indicate restored hippocampal function underlies the improvement of MWM performance of animals under the same lighting regimen (Figure 1.2). It should be noted that the animals in the present study were all young adults (4–6 months old). Whether this level of plasticity is retained in older animals and how aging may impact the modulatory effects of light on hippocampal function requires further investigation.

It has been proposed that most excitatory synapses are located at dendritic spines [95, 160], and their retraction or generation may underlie the neural mechanisms for learning and memory [161]. A more detailed morphometric analysis on CA1 apical dendritic segments revealed a significant reduction of stubby and mushroom spines in dimLD compared to the brLD group (Figure 1.5). Various studies demonstrate that after tetanic stimulation or behavioral training engaging the hippocampus, the spine apparatus prevalent in mushroom spines [162] recruits a wide array of molecules that enhance synaptic plasticity [93, 163, 164]. Therefore, the observed lower number of CA1 mushroom spines may reflect a degradation of synaptic plasticity that is correlated with impaired performance in the MWM. The changes in BDNF expression,

dendritic spine density, as well as spine morphology within the CA1, collectively suggest that ambient light modulates structural plasticity in the hippocampus.

The functional and structural changes in the hippocampus support the hypothesis that long-lasting changes in the brain, beyond temporary enhancement of arousal or attention, contribute to the superior cognitive performance associated with brighter illumination. Consistent with this hypothesis, seasonal variation has been reported in human cognitive brain responses, measured by P300 event-related brain potentials [165, 166] and fMRI [24]. And the P300 amplitude has been shown to be influenced by seasonal variation in the available amount of sunshine [166]. Enhanced cognitive function by light has traditionally been explained in reference to sleep and circadian rhythms [104], and sleep and circadian regulation certainly play a role in memory and hippocampal functions [167, 168]. However, lighting conditions can also influence learning/memory through circadian-independent mechanisms likely to involve melanopsin-based photoreception [105]. Our results provide evidence that lighting condition modulates the functional connectivity of the neural circuit within the hippocampus. More work is required to further elucidate the neural pathways mediating the effects of ambient light on the hippocampus. A possible candidate would be the hypothalamic orexin/hypocretin neurons, which have been implicated in many important functions including wakefulness, energy homeostasis, emotion, and cognition [65, 169]. Our previous work in grass rats has shown that the number of orexin-ir neurons and the density of orexin-ir fibers are affected by lighting conditions, with higher levels of orexin-ir in brLD than in dimLD groups [103]; and orexin pathways mediate the effects of light on other brain regions, i.e., the dorsal raphe [132] and hypothalamic dopaminergic neurons [121]. Orexinergic cells project directly to the hippocampus in both nocturnal laboratory rats and diurnal grass rats [70, 170]. Thus, the orexin system is well

positioned to mediate the effects of light on hippocampal-dependent learning and memory, an idea that will be further explored in future studies.

The present study is a first step towards a better understanding of how ambient light modulates cognitive functions in diurnal species. Such knowledge is significant for the design of lighting environments that promote optimal cognitive function. In the United States, a majority of the population spends ~90% of their time indoors, where the lighting is less bright than outdoors [44]. Even in optimal environments, light deficiency can occur as a result of reduced ocular transmission related to retinal disease or aging [171-175]. Although light pollution or light exposure at night has recently been recognized as a negative factor for ecology and human health [176, 177], the consequence of insufficient light during the day has received less attention. Our finding that 4 weeks of daytime light deficiency leads to a reduction in the functional connectivity within the hippocampus and to impairments in spatial learning and memory underscore the salient effects of light on our brain and behavior. A mechanistic understanding of the effects of light on cognition can help to identify risk factors for cognitive decline and contribute to the development of more effective prevention and treatment of cognitive impairment in clinical populations.

CHAPTER 3:

Daytime light intensity modulates spatial learning and hippocampal plasticity in female Nile grass rats (*Arvicanthis niloticus*)

The work presented in this chapter has been published in manuscript form.

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Introduction

The day/night light-dark cycle is the most reliable and predictable cue in the environment influencing our brain and behavior. For diurnal mammals including humans, bright light during the day entrains circadian rhythms, promotes wakefulness and arousal, and influences emotion and cognition [3, 31, 32]. Insufficient daylight exposure has a negative impact on emotion and cognition, as seen in patients suffering from seasonal affective disorder (SAD), in which the typical symptoms include depression, anxiety, low motivation and cognitive impairment [18]. The onset and remission of the symptoms in the fall and spring, respectively, is associated with seasonal fluctuation in the amount of sunlight that individuals receive. Before its full remission in the spring, the SAD symptoms can be alleviated by bright light therapy, further supporting the notion that the cause of SAD is light deficiency. In addition to the changes in ambient light, reduced photoreception can result from eye diseases, such as glaucoma or age-related macular degeneration, which have been associated with cognitive impairments [178, 179]. The impact of light on cognitive function has also been documented in non-clinical populations independently of seasonal changes in photoperiod, as brighter light has been found to improve standard test scores of school children and productivity at work [26, 27, 107, 108]. These findings collectively suggest that the amount of daylight is positively related with superior cognitive function, but the underlying mechanism for this effect of light is unclear.

Our previous work utilized a diurnal rodent model, the Nile grass rat (*Arvicanthis niloticus*) to study the effects of ambient light on hippocampal function [180]. We found that male grass rats housed in dim light during the day had impairments in the Morris water maze (MWM), a spatial learning/memory task, compared to those housed in bright light, and that the behavioral deficits were accompanied by reduced brain-derived neurotrophic factor (BDNF) and

dendritic spine density in the hippocampus. The results suggest that ambient light modulates spatial learning and memory through structural and functional changes within the hippocampus. Since these findings pertain to only males, whether or not light affects female grass rats in a similar manner remains unknown. Although it might be intuitive to expect female grass rats to show the same behavioral and hippocampal responses following the same light paradigm, that is not necessarily our hypothesis, as males and females differ in various brain functions including learning and memory [181-183]. Particularly, sex differences in spatial learning and memory have been documented in numerous research efforts using humans and rodents [184, 185]. In humans, consistent sex differences favoring males have been found in virtual MWM tasks, route learning and spatial rotation [183]. In rodents, the results are less consistent, with sex differences found in some studies but not in others [183]. For the MWM task, a sex difference was detected with a male advantage for rats, and a small female advantage in mice. Many factors can influence the sex differences in spatial memory tasks. In rats, the male advantage in MWM was greater when the animals were raised in isolation or without receiving pre-training trials [185], suggesting a possible interaction between sex and social stimulation and/or sex and stress, such that environmental, social, and experiential factors affect learning and memory in a sex-specific way. Therefore, it is possible that the ambient lighting conditions may also have different impacts on the cognitive performance of each sex.

The objective of the present study is to expand our previous findings on male grass rats by investigating the effects of light on hippocampal function in female grass rats, and to explore potential sex differences in behavioral and hippocampal structural and molecular responses following chronic daytime light deficiency. The majority, if not all, of the rodent studies on sex differences in learning and memory use nocturnal species, i.e. laboratory rats or mice. Diurnal

and nocturnal species have adapted to different temporal niches through the entrainment of their circadian system by light as well as by showing opposite responses to acute presentations of light, to achieve optimal behavioral competence during the day or night, respectively [32, 186]. Our studies on spatial learning and memory in a diurnal animal model under different light intensities during the daytime, the active phase of the species, will fill a gap in the literature and provide insight into how ambient light modulates hippocampal function for both sexes in diurnal mammals, like humans.

Experimental Procedures

Subjects

Female unstriped Nile grass rats (*Arvicanthis niloticus*) used in all experiments were obtained from the breeding colony at Michigan State University. The animals were initially housed in a 12:12 hr light-dark (LD, ~300 lux during the day) cycle in plexiglass cages (34×28×17 cm) with food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and water available *ad libitum*. During the experiment, animals were housed under either a 12:12hr bright light-dark (brLD, ~1000 lux during the day) or dim light-dark (dimLD, ~ 50 Lux) cycle as in our prior studies [47, 103, 180]. Fluorescent light fixtures (Jesco Lighting, SP4-26SW/30-W) were utilized for the behavioral experiment, four cabinet lights (two in the front and two in the back) were attached to the top level of every row within a cage rack. The color temperature for these fixtures was approximately 3,000K. For enrichment, a PVC tube was provided in the cages. This also served as a hut for the grass rats to hide, thus direct light exposure during the experiment was voluntary.

Morris Water Maze

Female grass rats (n=8/lighting condition) were used in this experiment. Animals were singly housed in either brLD or dimLD for 4 weeks prior to being trained on the Morris Water Maze (MWM). During the 4th week of housing in each lighting condition, the animals were handled daily for 10 minutes in their home cage in the behavioral testing room. In the following week, animals were trained and tested for MWM. The handling, training and testing was performed the same way as described in a previous study using male animals [180]. Animals were trained and tested during *zeitgeber* time (ZT, ZT0 is lights on) 5-7; the light intensity in the testing room was ~ 300 lux. Training on the MWM was performed using a circular pool (60 cm depth x 122 cm diameter) with a platform (15-cm diameter) located 2cm under the water level and approximately 30cm away from the perimeter of the pool. The water was made opaque with non-toxic white paint and kept at 26±2^{o}C, with different geometrical cues posted up on each wall in the room for spatial orientation. In order to be certain of normal motor abilities, the animals performed one-day cued-platform training in which the platform was visible before exposure to the hidden-platform training [123]. All animals tested in the task located the platform in less than two minutes when it was visible. In the following 5 days training session, two training trials were completed each day with each trial at a maximum of two minutes in length with a 30-second inter-trial interval. Animals that failed to locate the platform in the 2-minute period were guided in the direction of the platform and given a latency of 120 seconds. Twenty-four hours after the final training session, reference memory was tested with the platform removed from the MWM and each grass rat swam for one minute. Training and testing sessions were recorded and analyzed using Noldus *Ethovision* (*XT* 8.5, Noldus Information Technology, Netherlands) by an experimenter who was blind to the experimental conditions.

Morphometry

Behaviorally naïve female grass rats (no experience with training or testing) were housed under either brLD or dimLD for 4 weeks prior to bilateral injection of herpes simplex virus expressing green fluorescent protein (HSV-GFP, Harvard Massachusetts General Hospital Viral Core Facility) into the dorsal hippocampus. The surgical procedure, histology and confocal microscopic analyses were performed as in the previous study using male grass rats [180]. In brief, 0.5µl of HSV-GFP was infused at a rate of 0.1µl/minute at the following coordinates from bregma: -0.1mm A-P, ± 2.0 mm L-M, and -2.7mm D-V from the surface of the brain. Animals were perfused transcardially 48h post-surgery. Brains sections at 100 µm thickness were mounted onto subbed glass slides with ProLong® Diamond Antifade Mountant (ThermoFisher Scientific, Waltham, MA), and were examined using a Nikon A1Rsi laser scanning confocal microscope at 1000x magnification. A z-stack was obtained for each sample to observe detailed morphology of dendritic spines, and was reconstructed to three dimensions using the NeuronStudio freeware morphometric program utilizing the rayburst algorithm [135]. Five neurons (two dendritic segments/neuron) were analyzed per animal. Dendritic segments were randomly chosen 50-150 µm away from the soma for analysis; segments included were ~1.5µm in diameter for both groups, with even viral expression and no overlap with neighboring dendrites. Dendritic spines were classified on three parameters: (1) presence or absence of a neck; (2) head diameter; and (3) head/neck aspect ratio [96, 136]. Both thin and mushroom subtypes have visible necks, but where the mushroom subtype has a head which has a markedly larger diameter compared to the neck, the thin subtype does not have a notable difference between the head and neck diameter. The stubby subtype has a large head, but lacks the presence

of the neck. Each subtype of dendritic spines was analyzed from 20µm segments of two distinct dendritic branches per neuron.

Immunohistochemistry (IHC)

Animals tested in the MWM were left undisturbed for two days, and then they were transcardially perfused at ZT 5-7 with saline followed by 4% paraformaldehyde. Brains were processed for BDNF IHC followed by quantitative analysis following the same procedures as described in our previous study using males [180]. Briefly, 3 alternate sets of 40µm sections were collected; one set of 10 sequential sections containing the dorsal hippocampus was incubated with anti-BDNF primary antibody (1:5000, ab101747, Abcam, Cambridge, UK). The signals were visualized using 3,3'-Diaminobenzidine (DAB) and 4% Nickel Sulfate. BDNF-immunoreactive (ir) cells were counted on photomicrographs of the dorsal hippocampus by an experimenter who was blind to the experimental conditions. BDNF-ir cells were only counted if they exhibited immunoreactivity in both the nucleus and cytoplasm of the cell, those that only showed partial immunoreactivity were not included in the counts. Cells were counted within the CA1, CA3 and Dentate Gyrus (DG) subregions of the hippocampus with a 200µm x 400µm counting box [180].

Western Blot

Behaviorally naïve female grass rats were used for Western blot analysis. The animals were housed in either brLD or dimLD for 4 weeks prior to brain tissue collection at ZT 5-7 following decapitation. Flash frozen brains were sectioned coronally at 200 µm thickness, thaw-mounted onto a slide. The CA1 subregions were punched out from the slice using a 1-mm (diameter) micropuncher (Harris Micropunch, Hatfield, PA) and stored at -80 °C. For the analysis of BDNF (n=8/condition), tissue punches were then homogenized in radio-

immunoprecipitation assay (RIPA) lysis buffer (sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA) for protein extraction according manufacturer's instruction. Protein concentrations were measured with the Bradford assay method (Bio-Rad; Hercules, CA). From each animal, 20 µg total protein was run on precast gels (4-20% Tris-Glycine Mini; NuSep, Germantown, MD) and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks; Invitrogen by Thermo Fisher Scientific). Membranes were treated with REVERT Total Protein Stain Kit (P/N 926-11016; LI-COR, Lincoln, NE) to quantify total protein for western blot normalization using LI-COR Odyssey CLx Imaging System. After total protein imaging, membranes were washed with REVERT reversal solution to remove total protein stain and the membranes were proceeded for BDNF immunoblotting. Membranes were incubated in Odyssey Blocking Buffer TBS (OBB-TBS) on shaker for 1 hour at room temperature, followed by incubation in guinea pig anti-BNDF primary antibody (1:1000; AGP-021; Alomone Labs, Jerusalem, Israel) at 4 °C for 5 days. The BDNF antibody that was utilized detected both mBDNF and proBDNF, as well as any potential dimers or tetramers of BDNF. The membranes were then incubated with a IRDye 800CW secondary antibody (1:10,000, P/N 925-32411, LI-COR, Lincoln, NE). The fluorescence intensity of proBDNF (~35KD) and mature BDNF (mBDNF, ~16KD) were detected and quantified by LI-COR Odyssey CLx Imaging System and normalized to the total protein fluorescence intensity measured from the same animal. The ratio of fluorescence intensity for proBDNF or mBDNF over total protein of each animal were calculated and used for statistical analysis. A second cohort of female grass rats (n= 5-6/condition) were used for analyzing tropomyosin receptor kinase B (TrkB) and its phosphorylation at the tyrosine 816 (Tyr816) site (pTrkB). CA1 tissue punches were collected as for BDNF assay above. To preserve phosphorylated sites, tissue punches were homogenized in RIPA buffer with phosphatase

inhibitors (PhosSTOP™, Millipore Sigma). 100 µg total protein from each animal was loaded on the gel and was analyzed following the same procedures as for BDNF above. A rabbit anti-phospho-TrkB (Tyr816) (1:500, Millipore, Cat#ABN1381) was used to first detect the pTrkB (~140KD). Preadsorption was performed with control peptides to verify the specificity of the antibodies. The membrane was then stripped with Restore™PLUS Western Blot stripping buffer (Thermo Scientific, Ref# 46430) before being re-incubated with a rabbit anti-TrkB (~140KD; 1:1000, Alomone, Cat#ANT-019) to detect total TrkB (~140KD). For both pTrkB and total-TrkB detection, an IRDye® 680RD goat anti-rabbit IgG (1:10000, 0.1mg, LI-COR, cat#925-68071) was used. The phosphorylation ratio of TrkB was determined using the ratio of fluorescent intensity between pTrkB (Tyr816) and total TrkB.

Data Analysis

Statistical analysis was performed using SPSS (version 24, IBM, Armonk, North Castle, NY). For the MWM, the latency to reach the platform was analyzed within each trail using a 2 x 5 Mixed ANOVAs with lighting condition as the between–subjects factor and training days as the repeated measures factor. In the case that there was a significant interaction, repeated measure one-way ANOVA was used to analyze the effects of training days within each condition; when there was no interaction, only main effects were interpreted. Two-tailed independent samples student's t-tests were used to assess group differences on the amount of time spent in the goal quadrant, swim speed, and thigmotaxis (i.e. time spent swimming within 10cm of edge) during the probe tests. Dendritic spine density, the number of BDNF-ir cells and the level of mBDNF, proBDNF and TrkB phosphorylation ratio were compared between lighting conditions using two-tailed independent samples *student's t*-tests. The threshold for statistical significance for all analyses was established at $p < 0.05$.

Results

Chronic dim light housing impairs MWM performance of female grass rats

During the first trial over the 5 training days (Figure 2.1A), female rats in the brLD group located the platform more effectively when compared to those in the dimLD group (Figure 2.1A; main effect of training days: $F(4,56)= 8.332$, $p < 0.001$; main effect of lighting condition: $F(1,14)= 7.657$, $p < 0.05$). There was also a significant interaction between lighting condition and training days ($F(4,56)= 7.830$, $p < 0.001$). For brLD, the repeated measures ANOVA revealed a significant effect of training days ($F(4, 28)=14.361$, $p <0.001$); for dimLD, the training days had no significant effect ($F(4, 28)=1.29$, $p > 0.05$). During the second trial of each day (Figure 2.1B), which was conducted 30 seconds after the first one each day, both groups showed improved performance over training days ($F(4,56)= 7.914$, $p < 0.001$) without significant differences between the two groups (main effect of lighting condition: $F(1,14)= 1.181$, $p > 0.05$) or interactions between training days and light condition ($F(4,56)= 0.547$, $p > 0.05$). During the probe trial when the platform was removed from the pool, animals in the brLD group mainly focused their search within the target goal quadrant while those in the dimLD group displayed a random search pattern (Figure 2.1C). Quantitative analysis revealed that animals in the dimLD group spent significantly less time searching within the goal quadrant when compared to the brLD group (Figure 2.1D; $t(14)= 3.134$, $p < 0.01$), and the performance of dimLD animals was not different from the chance level of 15 seconds (one sample t-test, $t(7)= 2.03$, $p > 0.05$). Independent samples t-tests revealed that there were no significant differences between lighting conditions in either swim speed ($t(14)= 0.936$, $p> 0.05$) or thigmotaxis ($t(14)= -0.161$, $p > 0.05$).

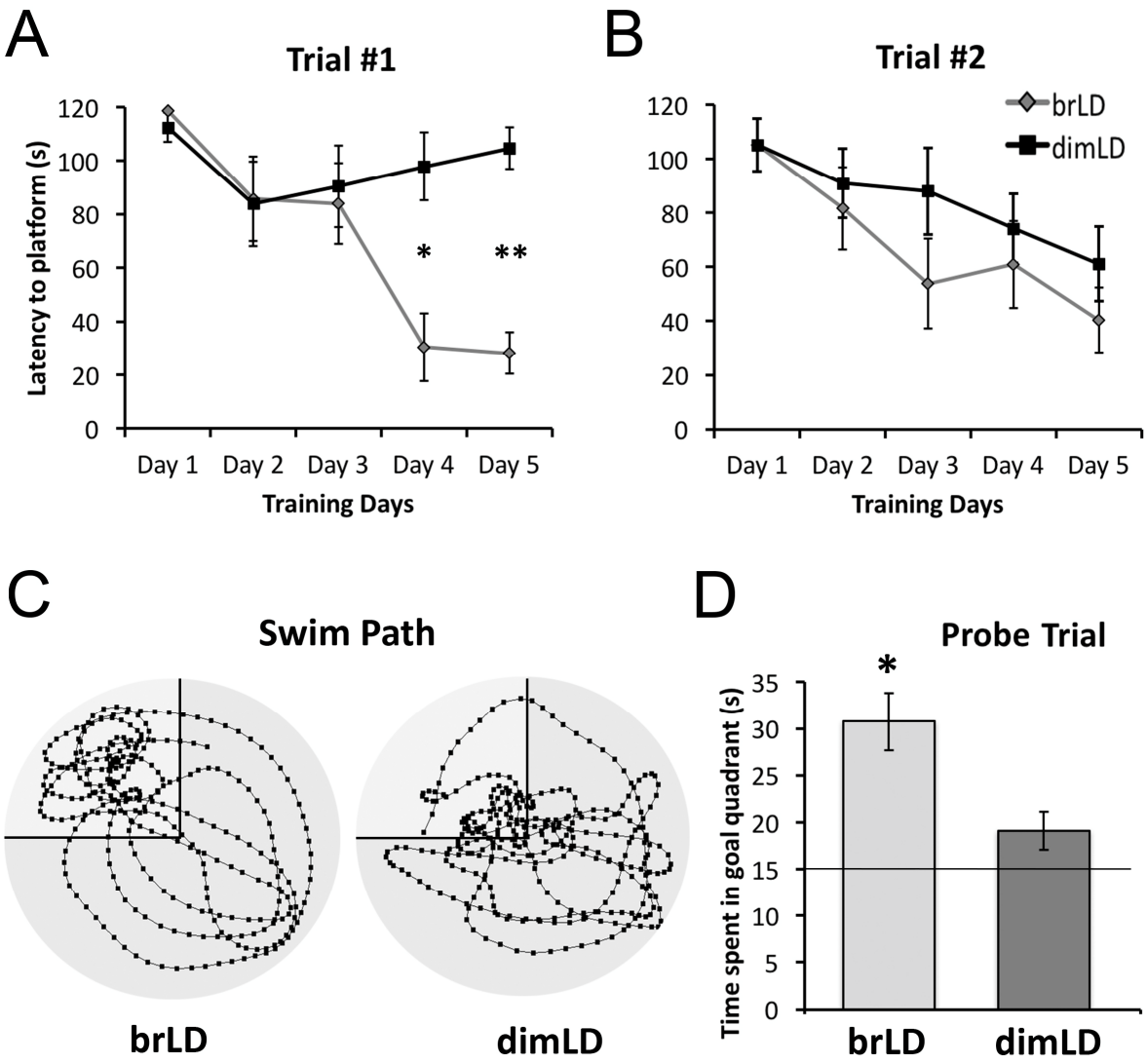

Figure 2.1 MWM performance of female grass rats housed in either brLD or dimLD conditions over 4 weeks. (A) Latency of the animals to find the platform in trial 1 (24-hour delay) over the course of 5 training days. Animals housed in brLD located the platform significantly faster than the animals housed in dimLD (main effect of training days: $F(4,56)= 8.332$, $p < 0.001$; main effect of lighting condition: $F(1,14)= 7.657$, $p<0.05$); interaction between lighting condition and training days ($F(4,56)= 7.830$, $p<0.001$). (B) Latency of the animals to find the platform in trial 2 (30-second delay) over the course of 5 training days. Both groups expressed improvement over the training days ($F(4,28)=14.361$, $p<0.001$) with no significant differences between the groups or interactions between training days and light condition. (C) Representative track plots of the search patterns used by brLD and dimLD animals during the probe trial (goal quadrant in red). (D) Grass rats in the dimLD condition spent significantly less time in the goal quadrant compared to grass rats in the brLD condition; horizontal line at 15 seconds indicates chance level performance. Data are shown as mean ± sem. *, $p < 0.05$

Chronic dim light housing leads to attenuated CA1 dendritic spine density in female grass rats

CA1 apical dendritic spine density was analyzed by each subtype (i.e. mushroom, thin and stubby) using 3D dendritic spine reconstruction from HSV-GFP transduced CA1 neurons (Figure 2.2A.) Quantitative analysis (Figure 2.2B) revealed a greater density of mushroom spines ($t(9)= 5.357$, $p < 0.001$) in the brLD compared to the dimLD group. There were no significant differences between the two groups in thin or stubby dendritic spines ($p> 0.05$). The total spin density of three subtypes combined is significantly higher in the brLD group ($t(9)= 2.879$, $p = 0.018$).

Photic modulation of hippocampal BDNF expression and TrkB phosphorylation in female grass rats

BDNF-ir in the hippocampus was comparable between female grass rats housed in brLD or dimLD condition (Figure 2.3A). Quantitative analysis revealed no significant difference in the number of BDNF-ir cells in CA1, CA3 or DG (Figure 2.3B, $p> 0.05$). The results were verified by Western blot in the CA1, there was no significant difference in the level of either mature BDNF or proBDNF between the two lighting conditions (Figure 2.4A, B, $p > 0.05$). Additionally, the phosphorylation of TrkB (Tyr816) in the CA1 was also not significantly different between females in either lighting condition (Figure 2.4C, D, $p > 0.05$).

Discussion

Using female Nile grass rats, the present study revealed that chronic dim lighting conditions (dimLD) resulted in impaired performance in the MWM, a hippocampus-dependent spatial task, along with reduced dendritic spine density in the CA1 subregion. Another marked difference was that in contrast to the downregulation of BDNF in dimLD compared to brLD in

males, the hippocampal BDNF expression remained unchanged in females exposed to the dimLD condition.

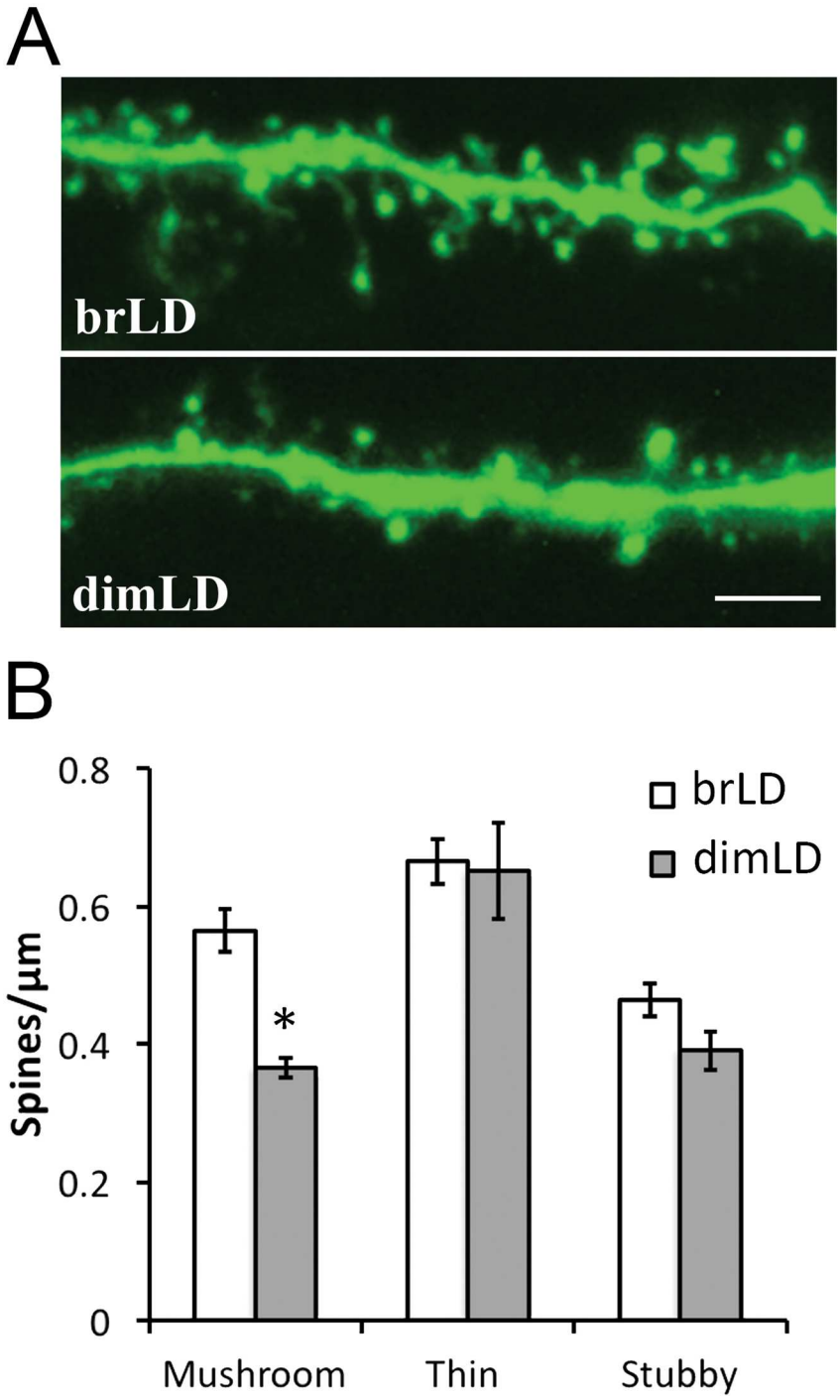

Figure 2.2 Hippocampal CA1 apical dendrites visualized and quantified by expression of HSV-GFP. (A) Visualization of dendritic spines using HSV-GFP expression. (B) Quantification of the dendritic density for each spine subtype. Scale bar, 5µm. Data are shown as mean ± sem. *, $p < 0.001$

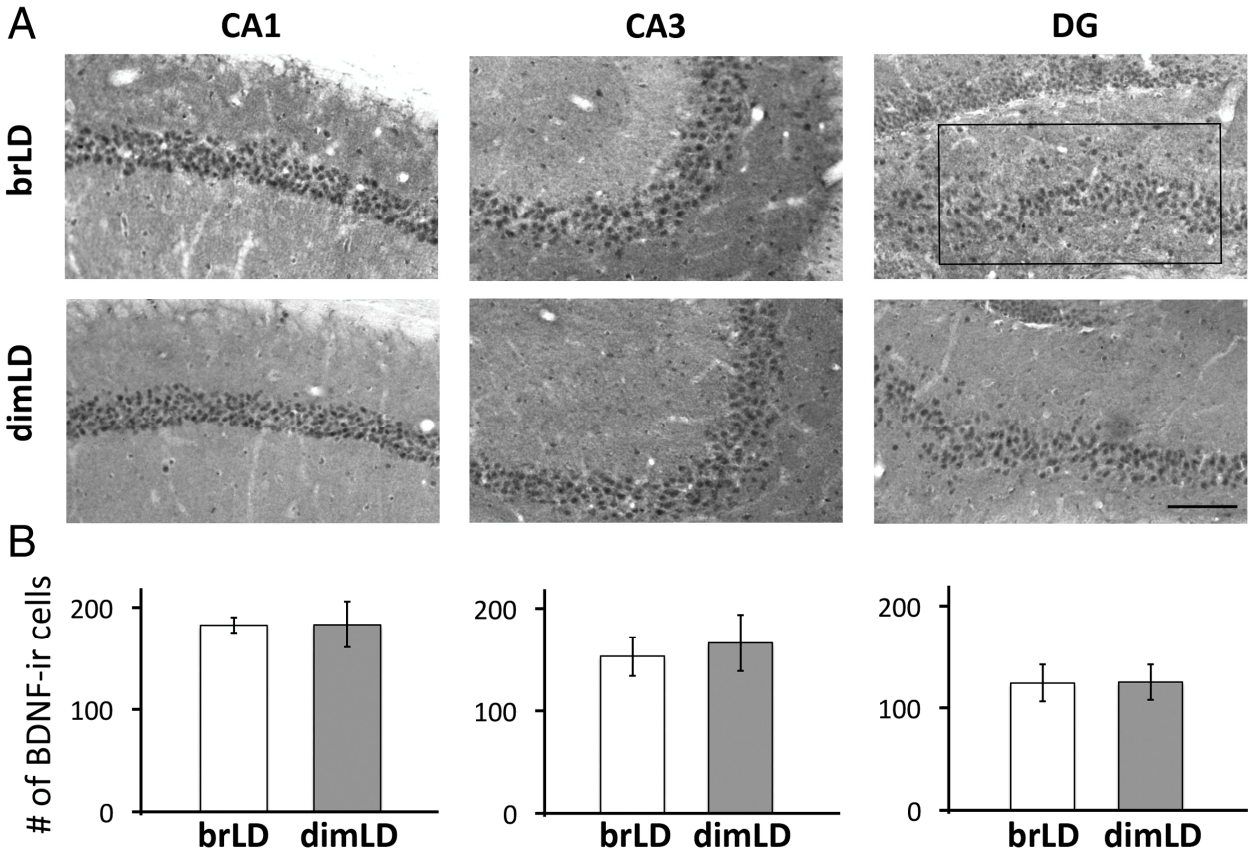

Figure 2.3 Hippocampal BDNF-ir cells in female grass rats housed in brLD or dimLD condition. (A) Representative photomicrographs of BDNF immunochemical staining in the CA1, CA3, and DG of the hippocampus in brLD and dimLD animals. (B) Quantitative analysis of the number of BDNF-ir cells in brLD and dimLD conditions in each hippocampal subregions. Data are shown as mean ± sem. Scale bar, 100 μm. The rectangle shows the size of counted area (200 μm × 400 μm). No significant differences were observed between the counts in brLD and dimLD condition in any of the subregion ($p > 0.05$).

Together, these results suggest that daytime light deficiency negatively impacts hippocampal function in both males and females, but through distinct neural mechanisms, with downregulation of BDNF being involved in males but not in females. Alternatively, a yet unknown mechanism common to both sexes and unrelated to changes in BDNF may be responsible for the effects of dim light on behavior and hippocampal morphology.

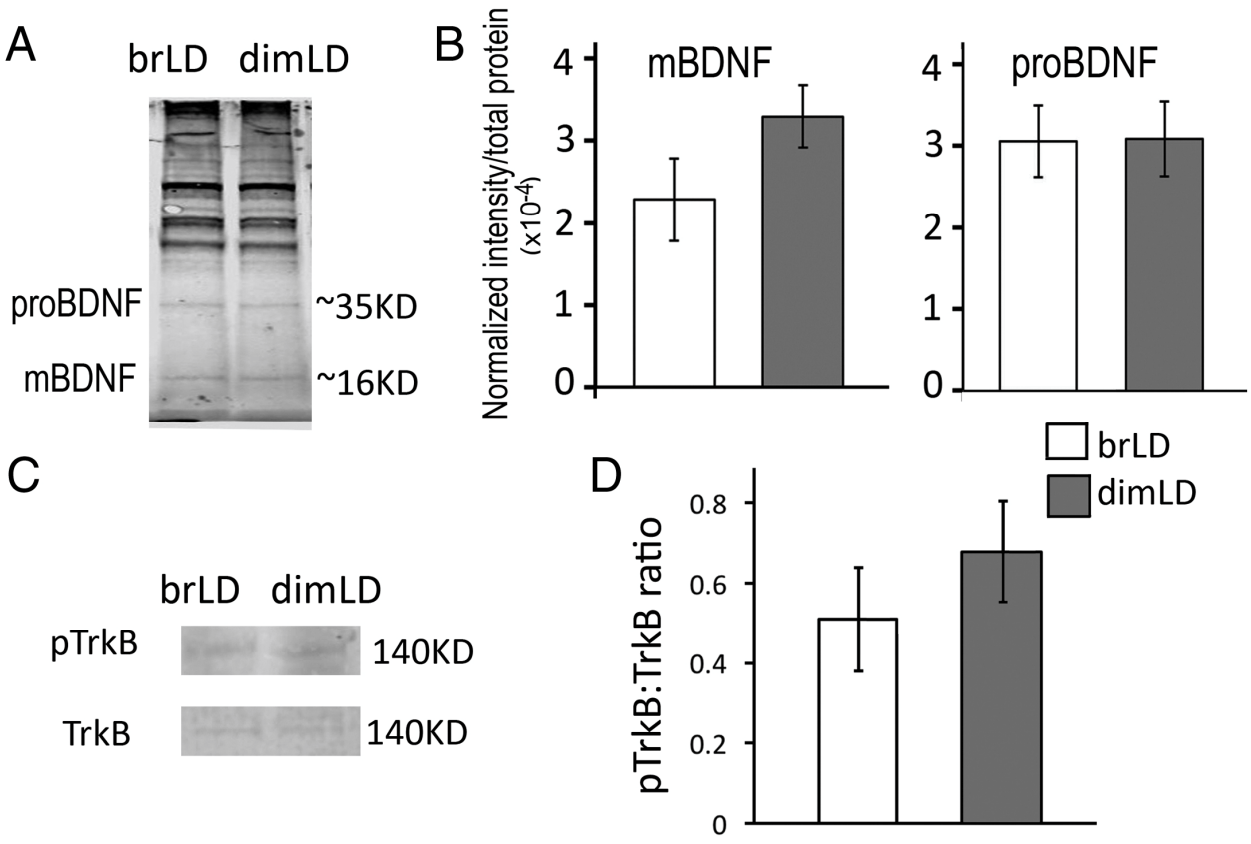

Figure 2.4 Hippocampal CA1 expression of BDNF and TrkB in female grass rats. (A) representative Western blot bands of mature BDNF (mBDNF) and proBDNF. (B) relative expression level of mBDNF and proBDNF. (C) representative Western blot bands of phosphor-TrkB at Tyr816 (pTrkB) and total TrkB. (D) phosphorylation rate of TrkB (Try816). Data are shown as mean ± sem. No significant differences were observed between brLD and dimLD in any of the measures ($p > 0.05$).

Female ovarian hormones have been shown to influence hippocampal functions, such that the strength of spatial learning/memory, CA1 dendritic spine density and BDNF expression all fluctuate with the estrous cycle [183, 187]. Female grass rats do not have spontaneous estrous cycles, and remain in diestrus when they are not co-housed with males [35]. Thus, there was no need to control for ovulatory cycle phases when they were trained and tested in the MWM task (Figure 2.1). Following a 30-second delay during the training session (trial 2, Figure 2.1B), the dimLD group performed similarly to the brLD group. This suggests that after completing the first training trial, animals in both groups encoded newly acquired information regarding the

location of the platform to perform the second trial effectively. However, the impairments of the dimLD group became evident when assessed after 24h delays. Analysis of the performance of the dimLD females across the first trials of the training phase, showed no evidence of learning (trial 1, Figure 2.1A). The absence of a learning curve in the dimLD group during trial 1seems to indicate that there was a lack of consolidation of newly acquired information because there is no progression in latency scores as training days go on. When reference memory was assessed, the dimLD females also performed at chance level during the probe trial (Figure 2.1D). If appropriate acquisition and consolidation of the MWM task occurred, animals would spend most of their time in the probe trial searching for the platform in the goal quadrant. By impairing the consolidation process during training, animals in the dimLD group exhibit poor reference memory by searching indiscriminately across all quadrants in the MWM.

The behavioral data seem to suggest that short-term memory (STM) is conserved and long-term memory (LTM) is impaired. A possible explanation for this occurrence may be that early phase of long-term potentiation (E-LTP) remains intact, but chronic daytime light deficiency may disrupt the induction and/or maintenance of late-phase LTP (L-LTP). STM is governed by E-LTP, which only depends on the activation of existing proteins and intracellular release of Ca^{2+} to traffic receptors and kinases to the synapse [188]. On the other hand, LTM relies upon altered gene expression and protein synthesis required for the persistence of L-LTP [189-191]. Therefore, it may be possible that chronic dim lighting conditions do not affect aspects of LTP that only consist of activating existing synaptic machinery, but impair those that are required for strengthening and stabilizing the synapse.

When the MWM performance of females in the present study was compared with male counterparts in our previous study [180], although the results are generally consistent, females in

dimLD seemed to exhibit greater behavioral deficits in the MWM task (Figure 2.5). When the MWM performance during the first trial of each training day was compared, animals were housed, females showed comparable performance as males in brLD condition (Figure 2.5A), but much worse performance in females compared to males in the dimLD condition (Figure 2.5B). In dimLD conditions, in contrast to the modest but significant improvement over training days seen in males, there was no improvement over training days in females. The difference between females and males in dimLD points to a higher vulnerability in females to the detrimental effects of chronic daytime light deficiency on a hippocampal-dependent memory task. This higher level of susceptibility in females may also apply to other challenges that affect hippocampal functioning and may account for the female-bias seen in some neurological or psychiatric disorders e.g., Alzheimer's disease and depression [192, 193].

Previous work using male grass rats revealed attenuated CA1 dendritic spine density, especially the mushroom and stubby type of spines following dimLD housing [180]. Consistent with those observations, female grass rats exposed to dim illumination showed fewer mushroom spines compared to their brLD counterparts (Figure 2.2). Spine morphology has been linked to the function and stability of a synapse. Mushroom and stubby spines have larger heads that are positively correlated with the size of the post-synaptic density (PSD) area and the number of NMDA and AMPA receptors and docked presynaptic vesicles [194-197]. Consequently, Ca^{2+} influx from NMDA receptors leads to the activation of calcium/calmodulin-dependent protein kinase II (CaMKII). With repeated high-frequency Ca^{2+} influx, CaMKII undergoes autophosphorylation that confers constitutive $Ca2^{+}$-independent kinase activity, and allows enhancement of AMPA

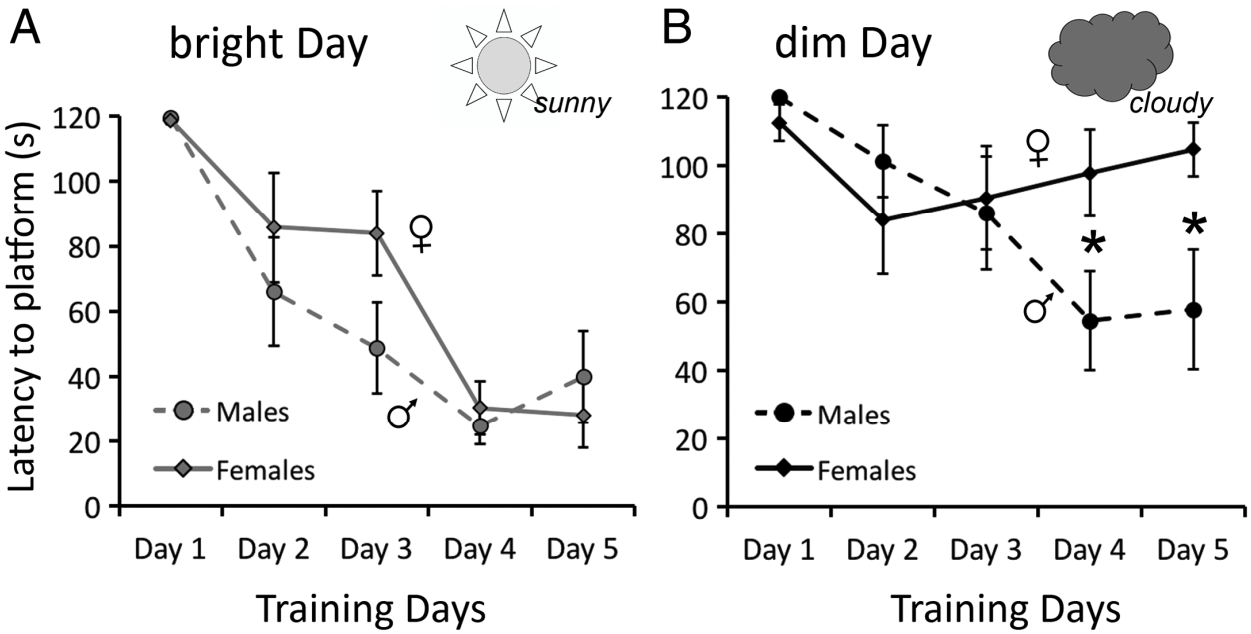

Figure 2.5 Sex differences in MWM performance following reduced daytime illumination. MWM performance in trial 1 (24-hour delay) over the 5 training days was compared between female and male grass rats. The female graphs are replotted with data shown in Figure 2.1, male graphs are replotted using data published in a previous study [180] with permission. (A) In brLD condition, females and males showed comparable performance in MWM task (main effect of sex: $F(1,14)=1.01$, $p > 0.05$; interaction between sex and training days: $F(4,56)=1.54$, $p > 0.05$). (B) In dimLD condition, males outperformed females in the MWM. There was a significant interaction between sex and training days ($F(4,56)=3.739$, $p < 0.01$). Post-hoc comparison revealed a significant difference on training day 4 and 5 (*, $p < 0.05$). A significant main effect of training days was present in males ($F(4,28)=1.29$, $p < 0.01$), but absent in females ($F(4,28)=1.29$, $p > 0.05$).

receptors to the synapse, critical mechanisms for LTP [198]. Additionally, enhanced CaMKIIα activity is restricted to dendritic spines that undergo enlargement [199]. The spine morphology of grass rats was analyzed in behaviorally naïve animals that were not trained in the MWM. Thus, the lower density of mushroom and stubby spines in the dimLD group indicates that there are less mature synapses available to engage memory formation even before the MWM training. Behavioral experience, i.e. training in the MWM, can induce neural plasticity, and leads to the development and/or maturation of spines corresponding to the strengthening of neural connections that are needed for memory formation [200]. Although not directly tested, it is

possible that compared to the difference seen in naïve animals (Figure 2.2), the differences in spine morphology between brLD and dimLD groups would be more salient following MWM training and testing, and could potentially reveal sex differences in spine morphology not seen in naïve animals here.

In contrast to the impaired spatial memory and attenuated hippocampal spine density, daytime dim light housing had no significant effect on hippocampal BDNF expression in female grass rats. There was no significant difference in the number of hippocampal BDNF-ir cells (Figure 2.3) or BDNF protein in CA1 (Figure 2.4) between females in dimLD and brLD conditions. On the other hand, a significant reduction of BDNF-ir cells [180] and BDNF in CA1 (data not shown) following dimLD housing was observed in male grass rats. To further analyze BDNF signaling, we also examined its high-affinity binding receptor, TrkB. Ligand-mediated phosphorylation of TrkB at Tyr816 leads to phospholipase-Cγ (PLCγ) mobilization of intracellular Ca^{2+} stores, a vital step for LTP maintenance [99]. However, no significant differences were observed in Tyr816 phosphorylation between the two lighting conditions. The results in BDNF and TrkB expression suggest that the modulation of daytime light intensity on hippocampal function may involve different signaling pathways in males and females. It should be noted that the analyses of BDNF and TrkB were conducted following 4 weeks of animals housed in each lighting condition, and thus the results do not obviate the possibility of changes in BDNF-TrkB pathways at earlier time points. Such earlier changes could contribute to the reduction in mushroom spines and the behavioral deficits in MWM, and by the time the structural and behavioral changes became evident, the BDNF-TrkB pathways in dimLD group could have reached the "new normal" steady state of the system, no longer showing significant differences from the brLD group. On the other hand, sex-specific responses in hippocampal

BDNF have been observed in other rodents following various paradigms/treatments. For example, isolation or maternal deprivation reduces BDNF expression solely in male but not female mice [201-203]; while enrichment induces more BDNF expression in females than in males [204, 205]. The findings from diurnal grass rats contribute to the existing literature on sex-specific regulation of hippocampal BDNF, and suggest the possibility of distinct neural mechanisms underlying the modulatory effects of ambient light on hippocampal function in males and females.

The cellular mechanisms or molecules responsible for deficits in hippocampal synaptic plasticity and spatial memory in female grass rats housed in dimLD in the absence of changes in BDNF remain unknown. In addition to BDNF, transcription factors of the nuclear factor-κB (NF-κB) family have been implicated in synaptic plasticity [206-208]. Many members of the NF-κB family activate the common intracellular kinase pathways that are also the target of BDNF, to execute synaptic strengthening in a similar fashion, and thus could be an alternative molecule regulated in female grass rats in dimLD. Furthermore, astroglial NF-κB has been shown to play a sex-specific role in learning and memory, such that overexpression of NF-κB inhibitor in transgenic mice leads to impairments in hippocampal-dependent tasks in females without affecting males [209]. Future studies will test the possible involvement of the transcription factors of the NF-κB family in photic modulation of hippocampal functions in grass rats.

The present study expanded our previous findings on the effects of light on the hippocampal functions in male diurnal grass rats, to show that the daytime dim light condition leads to impaired hippocampal function in females as well, but with more salient behavioral deficits in females compared to males. Furthermore, the results suggest that ambient lighting

conditions activate sex-specific neural responses within the hippocampus to modulate spatial learning and memory as well as hippocampal dendritic spine morphology. For humans, the amount of light we are exposed to varies over the seasons and over the life span, with older people being particularly at risk for insufficient light exposure. Older adults living in residential care homes experience a mean daytime light exposure that is less than 500 lux [210, 211]. In a group of individuals 60-100 years old, the median duration of light above 1,000 lux that they experienced was only 9 min a day, and within that group, for those with advanced cognitive decline, this duration dropped to 1 min [210, 211]. Given our findings in the grass rat model, it is reasonable to expect that the low level of illuminance and the extremely short duration of bright light will impair the already fragile hippocampus of the aging brain and accelerate aging-related cognitive decline. Furthermore, the prevalence of dementia and Alzheimer's Disease is higher in women than in men [192, 212], suggesting a possible sex differences for humans with respect to vulnerability to environmental challenges, which may include light deficiency. A better understanding of the neural mechanisms underlying impaired learning and memory in both males and females will contribute to gender-specific strategies for the prevention and treatment of cognitive impairments.

CHAPTER 4:

Light, Orexin, and Hippocampal Function

The work presented in this chapter is currently being prepared for submission to a peer-reviewed journal.

Introduction

As an environmental stimulus, light has a profound impact on several biological processes including cognitive function. Long-term bright light therapy has shown to improve academic performance in elementary schoolchildren [25, 107], increase working productivity in adult office employees [109, 213] and slow down cognitive decline in elderly dementia patients [29]. An environmental factor that contributes to reduced light exposure is the seasonal fluctuations of natural light intensity, with spring and summer boasting higher illuminance levels when compared to fall and winter. In addition to this, the average person spends approximately 90% of their time indoors [44], which can further limit bright light exposure and bring about diminished cognitive performance. Performance in sustained attention tasks are sub-optimal in healthy adults when tested during the winter solstice in comparison to testing in the summer solstice [24], and patients suffering from Seasonal Affective Disorder (SAD), a depressive disorder linked to seasonal fluctuations of light intensity [214], exhibit lower scores in cognitive tasks when depressive symptoms are present during fall and winter while displaying higher scores during symptom remission in the spring and summer [21, 22]. However, empirical evidence on the neurobiological underpinnings of how light influences cognitive function is relatively scarce.

The most commonly utilized mammalian animal models for research in the life sciences are domesticated/inbred strains of mice (*Mus musculus*) and rats (*Rattus norvegicus*). These traditional models have been used extensively due to physiological similarities with humans, and because of their lower cost for maintenance and breeding. A limitation for these animals is their nocturnal activity pattern being opposite from the diurnal pattern exhibited by humans. Considering that most biological processes display circadian rhythmicity and that an organism's

chronotype (diurnal vs. nocturnal) dictates differing physiological states in both the brain and behavioral measures during the day and night [31], a diurnal animal model would be more suitable to study the neural mechanisms of how light influences human cognition. Several diurnal animals have been utilized in laboratory settings, they include but are not limited to, the Mongolian gerbil (*Meriones unguiculatus*), the degu (*Octodon degus*), and the Nile grass rat (*Arvicanthis niloticus*). Of the previously mentioned species, the Nile grass rat displays the highest diurnality index (0.87) [38] with the total duration of its active phase (13.5hrs) being closest to that of humans (15.5hrs) [39]. Although recent findings have demonstrated that the antelope ground squirrel (*Ammospermophilus leucurus*) displays a higher diurnality index (0.97), the Nile grass rat has a more complete track record of neuroanatomical and neurophysiological research efforts that makes more suitable as a model of mammalian diurnality.

Our previous research has focused on determining the effects of reduced daylight intensity on cognition rather than those of daylight duration because in our modern world, artificial lights reduce the variability of length of light exposure in humans. Further, the duration of light exposure above 1000lux is significantly reduced in wintertime when compared to summer [215]. Our lab has established a chronic daytime light deficiency paradigm using African grass rats, this paradigm consists of exposing animals to either 12:12hr light-dark (LD) cycle with bright light (brLD: 1000lux) or dim light (dimLD: 50lux) for a duration of four weeks. Animals in both conditions of this paradigm are able to entrain to their respective LD cycles regardless of brightness during the light phase [47]. These findings provide a first line of evidence that light's modulation of cognitive function may be a product of a direct effect that it exerts on brain regions that are essential in cognition. However, because there is no available data on how this lighting paradigm impacts other circadian parameters such as phase duration

and phase amplitude, circadian-dependent mechanisms cannot be discarded from playing a role in light-modulated cognitive function. The hippocampus (HPC) is a brain region vital to learning and memory [49], and it has been demonstrated that light elicits functional activation within this region [111]. A previous study [180] from our lab found that when grass rats are exposed to four weeks of dimLD conditions they exhibit impaired hippocampal function that is associated with poor performance in a spatial navigational task i.e., the Morris Water Maze (MWM) [131]. Additionally, that study found that the expression of hippocampal brain-derived neurotrophic factor (BDNF), a protein involved in hippocampal synaptic plasticity and long-term potentiation (LTP) [216], and dendritic spine density were both reduced in the CA1 subregion of the dorsal HPC (dHPC). Parallel to these findings, the study found that orexin-A (OXA) was drastically reduced in the lateral hypothalamic area (LHA) following four weeks of dim LD exposure [103]. In recent years, the evidence of orexin playing a role in HPC-dependent learning and memory has increased, this ranges from results showing that activating and inactivating orexinergic signaling pathways influence behavior [75, 76, 217, 218] and how showing that infusing cultured hippocampal slices with OXA induces an increase in cell firing rates [77]. Therefore, we hypothesize that light influences HPC-dependent learning and memory through the orexinergic system.

To investigate the role of OXA in light-modulated hippocampal function, our approach consisted of bi-directional manipulations of the orexinergic system. Our first manipulation was to test use intranasal administration of OXA to prevent hippocampal deficits induced by chronic daytime light deficiency in diurnal grass rats. For our second manipulation, we induced a viral vector-mediated knockdown of orexin-1 receptors (OX1R) within the dHPC in an attempt to induce the behavioral phenotype as we have seen previously in chronic dim lighting conditions,

but now in animals kept under brLD. We assessed HPC-dependent learning and memory as well as anatomical and molecular correlates that are involved in synaptic plasticity. In this study, we demonstrate that intranasal administration of OX-A helps ameliorate the negative effects chronic dim lighting conditions on learning and memory by increasing the rate of phosphorylation for proteins involved in hippocampal synaptic plasticity. Additionally, we show that OX1R knockdown within the Cornu Ammonis 1 (CA1) subregion of the dorsal HPC (dHPC) results in impaired MWM performance along with reduced hippocampal BDNF expression and CA1 dendritic spine density.

Experimental Procedures

Animals

Male grass rats 4 to 6 months were obtained from our breeding colony at Michigan State University. Prior to the experiments, animals were group-housed in a 12-hr light-dark (LD) cycle (lights on 7:00 AM; ~300 lux at the center of colony room). Food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and water were available *ad libitum*. During the experiments, animals were singly housed in either a bright light-dark (brLD; 1,000 lux at cage level) or a dim light-dark cycle (dimLD; 50 lux at cage level) for 4 weeks as in our previous studies [47, 103, 180]. A PVC tube and a cotton square were provided in each cage for enrichment purposes. The PVC tube also served as shelter for the grass rats to hide, thus direct light exposure during the experiment was voluntary.

Intranasal orexin A (OXA) peptide administration

Grass rats housed in dimLD were used in this experiment. During the 4th week of dimLD housing, animals were handled daily for 7 days to acclimate them to an intranasal grip, i.e. placing the animal in the palm of hand with their ventral side facing up, that would allow for

administration with minimal stress [219]. During the following week, animals received 6 µl of either OXA (5 nmol; California Peptide Research, cat#471-99) or vehicle solution (sterile pH-balanced 0.9% saline) intra-nasally (IN) following procedures modified from a previous study [219]. The OXA and vehicle solutions were administered 90 minutes prior to Morris Water Maze (MWM) training over the 5 training days before testing reference memory with the probe test (n=8/treatment) (Figure 3.1A). During each training day for MWM, 6µl of solution was applied bilaterally to the rhinarium of an awake animal that was immobilized in the intranasal grip. The solutions were delivered 1µl at a time by alternating each side, and were allowed to dry between each drop and prior to returning the animals to their home cages. An initial dosage of 10 nmol was tested per previous study [220], which resulted in enhanced anxiety-like thigmotaxic behavior (i.e. time spent "wall-hugging" in an attempt to escape rather than solving the maze), while 5 nmol of OXA- and vehicle-treated groups showed similar thigmotaxic behavior (Figure S3.1).

Virus Injection

One of two recombinant adeno-associated viruses (rAAVs): rAAV-OX1R-shRNA or rAAV-SC-shRNA were injected bilaterally (0.5 µl/side; 0.1 µl/minute) into the CA1 region of dorsal hippocampus (anterior -0.1 mm, lateral: ± 2.0mm, ventral -2.5mm; from Bregma) of grass rats (n=11/treatment). Following the injection, animals were housed in brLD for 4 weeks prior to MWM training and testing. Details on AAV and surgical procedure are available in *SI Materials and Methods* (See Appendix B). The site of injection and viral transduction were evaluated based on GFP expression after MWM. Out of the 22 animals that underwent surgery (n=11/treatment), seven were excluded from data analysis. Three animals from the rAAV-OX1R-shRNA were excluded due to a lack of GFP expression within the targeted region. In the rAAV-SC-shRNA

four animals were excluded either due to poor swimming ability or excessive thigmotaxic behavior that would be a confounding factor for behavioral results. A total number of 15 animals (n=7-8/treatment) were included in the final analysis.

Behavioral Analysis

MWM training and testing of grass rats were conducted as described in previous studies [131, 180]. In brief, hidden-platform training took place across 5 days between 12:00-14:00 PM (5-7 hours after light onset), light intensity in the test room was ~300 lux. Each training day had two 2-minute sessions with a 30-second inter-trial interval. 24 hours after the last training trial, the hidden platform was removed from the pool to assess reference memory in a 60-second probe trial. Further details are available in *SI Materials and Methods*.

Western Blot

For the animals treated with intranasal OXA or vehicle, once testing finalized, brains were collected 48h later and processed for Western blotting to assess Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα) (Thr286) and GluR1 (Ser831) phosphorylation as well as BDNF expression (n=8/treatment). To analyze the CaMKIIα (Thr286) phosphorylation, membranes were first incubated with a mouse anti-phospho-CaM Kinase II, α subunit, (Thr286) (1:1000, Millipore, Cat#05-533) followed by a IRDye® 800CW goat anti-mouse IgG_1-Specific (1:10000, 0.5mg, LI-COR, cat#926-32350). While for GluR1 (Ser831) phosphorylation, membranes were first incubated with a rabbit anti-phospho-GluR1 (Ser831) (1:1000, Millipore, cat#04-823) followed by IRDye® 680RD goat anti-rabbit IgG (1:10000, 0.1mg, LI-COR, cat#925-68071). After measuring the fluorescent intensity for each phosphorylated protein, the membranes were stripped with Restore™ Western Blot Stripping Buffer (ThermoFisher Scientific, Cat#21059), prior to the detection of total CamKII or GluR1 using a mouse anti-CaM Kinase II, α subunit

(1:1000, Millipore, Cat#05-532) or rabbit anti-GluR1 (1:1000, Abcam, cat#ab31232), respectively. For BDNF expression, membranes were incubated in guinea pig anti-BNDF primary antibody (1:1000; AGP-021; Alomone Labs, Jerusalem, Israel). The membranes were then incubated with a IRDye 800CW secondary antibody (1:10,000, cat#925-32411, LI-COR, Lincoln, NE). The fluorescence signal of each protein was detected at the expected size (CaMKIIα, 50kDa, GluR1, 106kDa, mature BDNF (mBDNF) at ~16kDa) and the intensity was quantified by LI-COR Odyssey CLx Imaging System and normalized to the total protein fluorescence intensity measured from the same lane on the membrane. The normalized intensity was used for further analysis. Further details are available in *SI Materials and Methods*.

Spine morphology

Following behavioral testing, animals injected with viral vectors were perfused transcardially and brains were processed for morphological analysis within the hippocampal CA1 subregion as described in previous studies [180, 221]. Briefly, 100μm hippocampal sections were examined using a Nikon A1Rsi laser scanning confocal microscope at 1000x magnification. A z-stack of 5 CA1 neurons (two dendritic segments/neuron) was obtained from each animal, and was reconstructed to three dimensions using the NeuronStudio freeware morphometric program. In the dendritic segments, three subtypes (e.g. thin, stubby, and mushroom) were identified based off the length of the neck and the diameter of the head [96, 136].

Statistical Analysis

All data analyses were carried out by an experimenter blind to the group identity of each animal. Statistical analyses were performed utilizing SPSS (version 24, IBM, Armonk, North Castle, NY). To analyze latency scores during the five-day training period in the MWM, a 2x5

mixed model ANOVA was used; training days was the repeated measures factor while treatment conditions (for both intranasal and viral vector experiments) was the between-subjects factor. When a significant interaction was detected, simple main effects were analyzed to determine the effects of training days within each condition. In the absence of a significant interaction, only main effects were interpreted. For MWM probe tests, morphometric analyses, and protein expression levels, independent samples *student*'s t-tests were used. The threshold of significance for all statistical analyses was established at $p<0.05$.

Results

IN OXA Improves Learning and Memory Despite Light Insufficiency when Administered Multiple Times during Training, but not a Single Administration following Training.

Prior to commencing the behavioral training experiments, we performed a dose-response study to determine what concentration of OXA (10nmol or 5nmol) would be suitable for IN administration. A one-way ANOVA ($F(2,17)= 21.022$, $p<0.001$) followed with a Games-Howell post hoc comparison (10nmol vs. vehicle and 5nmol, Figure S3.1) revealed that the 10nmol group exhibited approximately 3 times as much thigmotaxic behavior in comparison to controls and a low-dose group (5nmol). For the rest of the intranasal experiments, to avoid confounding results due to anxiogenic behaviors, a 5nmol concentration was utilized.

In the acquisition experiment, when comparing the first training trial of each training day (24h delay) between vehicle and OXA-treated groups a mixed-model ANOVA revealed a main effect of training days that indicated that both groups improved as training progressed (Figure 3.1B; n=8/condition; mixed model ANOVA; Main effect of treatment condition: $F(1,14)=2.566$, $p>0.05$; interaction: $F(4,56)=1.043$, $p>0.05$). However, no significant differences between groups nor interaction between groups and training days were found. Inversely, when both

groups were compared using data from the second training trial into consideration (30s delay), the OXA-treated group exhibited a faster learning curve in comparison to the vehicle-treated group (Figure 3.1C; mixed model ANOVA; Main effect of treatment condition: $F(1,14)=6.421$, $p=0.024$; interaction: $F(4,56)=0.903$, $p>0.05$). When the last day of training finalized, the hidden platform was platform was removed and reference memory was assessed 24h later. An independent samples t-test revealed a more consolidated search pattern in or around the goal quadrant in animals that were treated with OXA when compared to the vehicle group (Figures 3.1D and 3.1E; independent-samples *t*-test; $t(14)=3.297$, $p=0.005$). On average, OXA-treated animals spent approximately twice the amount of time searching inside the goal quadrant. No significant differences were present between groups regarding time spent exhibiting thigmotaxic behavior ($t(14)=-0.369$, $p>0.05$) nor swim speed ($t(14)=1.313$, $p>0.05$).

For the retrieval experiment, there were no group differences in latencies to locate the platform in the first trial (24h delay) throughout the training period from controls (See Appendix B, Figure S3.2B; mixed model ANOVA; Main effect of treatment condition: $F(1,14)=0.417$, $p>0.05$; interaction: $F(4,56)=0.427$, $p>0.05$). When the second trial was assessed (30s delay), the OXA group failed to distinguish itself from controls (Figure S3.2C; main effect of treatment condition: $F(1,14)=1.332$, $p>0.05$; interaction: $F(4,56)=1.193$, $p>0.05$). Following the training period, there were also no notable differences in reference memory between both groups (Figures S3.2D and S3.2E; independent-samples *t*-test; $t(14)=0.238$, $p<0.05$). No significant differences in swim speed ($t(14)= 0.78$, $p>0.05$) or thigmotaxic behavior ($t(14)= 0.238$, $p>0.05$) were observed for both groups.

IN OXA Increases CaMKII and GluR1 Phosphorylation when Administered Multiple Times during Training but not a Single Administration following Training.

Within the acquisition experiment, animals that were treated with IN OXA displayed a higher rate of CaMKIIα (Thr286) autophosphorylation within the dCA1 when compared to vehicle controls (Figure 3.2A; independent-samples *t*-test; CaMKIIα(Thr286)- $t(14)= -3.219$; $p= 0.006$; CaMKIIα- $t(14)= 0.358$; $p= 0.725$).

Consequently, CaMKIIα-mediated GluR1(Ser831) phosphorylation was elevated in OXA-treated animals in comparison to vehicle controls (Figure 3.2B; GluR1(Ser831)- $t(14)= -3.984$; $p= 0.001$; GluR1- $t(14)= -1.089$; $p= 0.295$).

Western blot analysis revealed no significant differences in CaMKIIα(Thr286) autophosphorylation upon IN administration of OXA during the retrieval phase of the MWM task (See Appendix B, Figure S3.3A; independent-samples *t*-test; CaMKIIα(Thr286)- $t(14)= -0.645$, $p>0.05$; CaMKIIα- $t(14)= -0.549$, $p>0.05$). Similarly, no significant differences were observed in GluR1(Ser831) phosphorylation (Figure S3.3B; GluR1(Ser831)- $t(14)= 1.052$, $p>0.05$; GluR1- $t(14)= 0.976$, $p>0.05$). The lack of significant differences of phosphorylation ratios of CaMKIIα(Thr286) and GluR1(Ser831) between vehicle controls and OXA-treated animals are consistent with the behavioral outcomes of animals in the retrieval experiment.

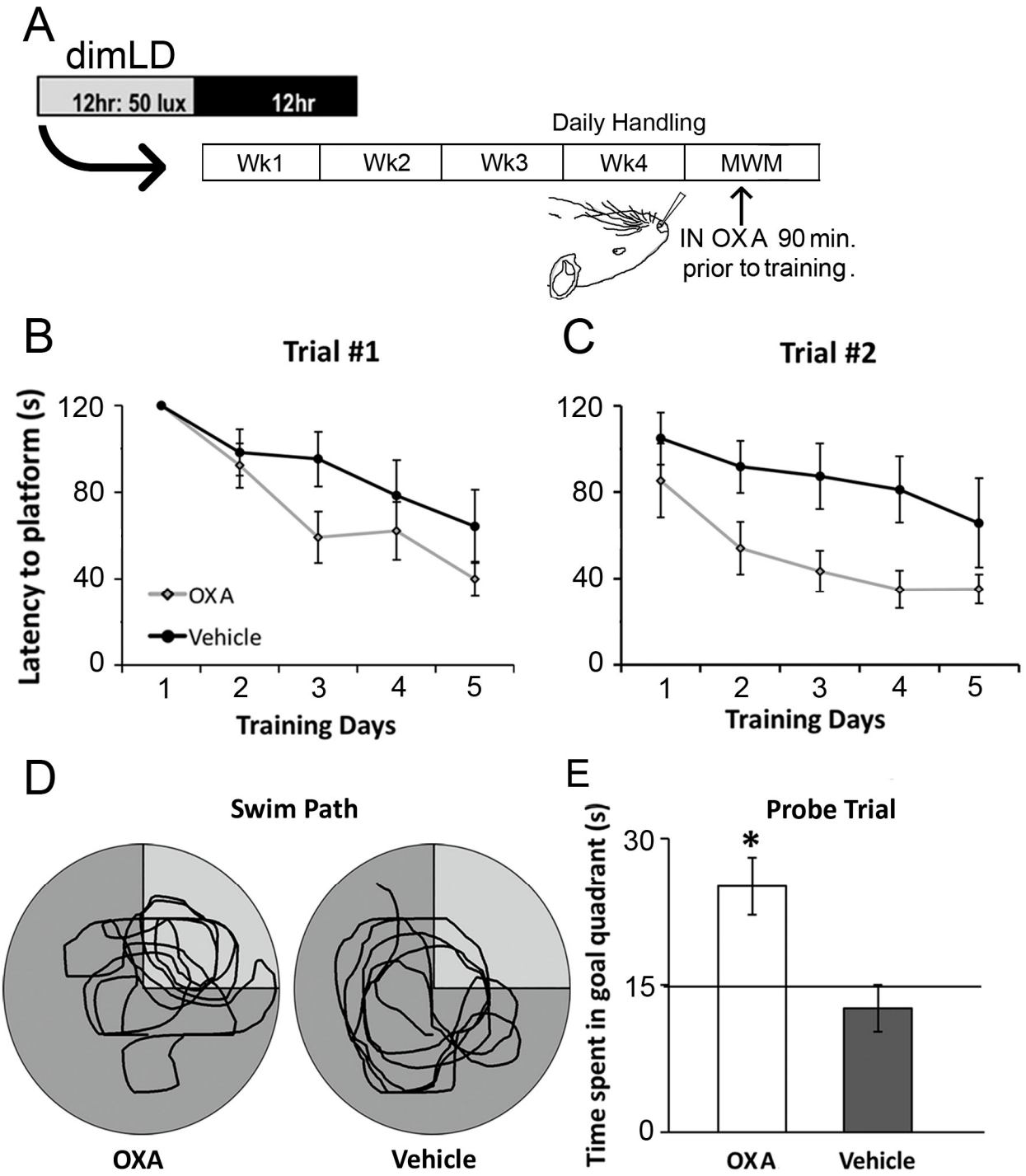

Figure 3.1 Multiple intranasal administration of OXA during training of MWM. (A) Four-week lighting paradigm along with the experimental timeline. (B) No significant differences emerged between both groups' latencies to locate the platform during the first training trial throughout the training period (n=8/condition; mixed model ANOVA; Main effect of treatment condition: $F(1,14)=2.566$, $p>0.05$; interaction: $F(4,56)=1.043$, $p>0.05$). (C) Animals in the OXA-treated group showed a lower latency to locate the platform during the second training trial throughout the training period (mixed model ANOVA; Main effect of treatment condition: $F(1,14)=6.421$, $p=0.024$; interaction: $F(4,56)=0.903$, $p>0.05$). (D) Representative swim paths for

Figure 3.1 Multiple intranasal administration of OXA during training of MWM (cont'd). animals in both lighting conditions. (E) OXA-treated animals spent more time in the goal quadrant during the probe test (independent-samples *t*-test; $t(14)=3.297$, $p=0.005$). Data are represented as mean ± SEM. *$p<0.01$.

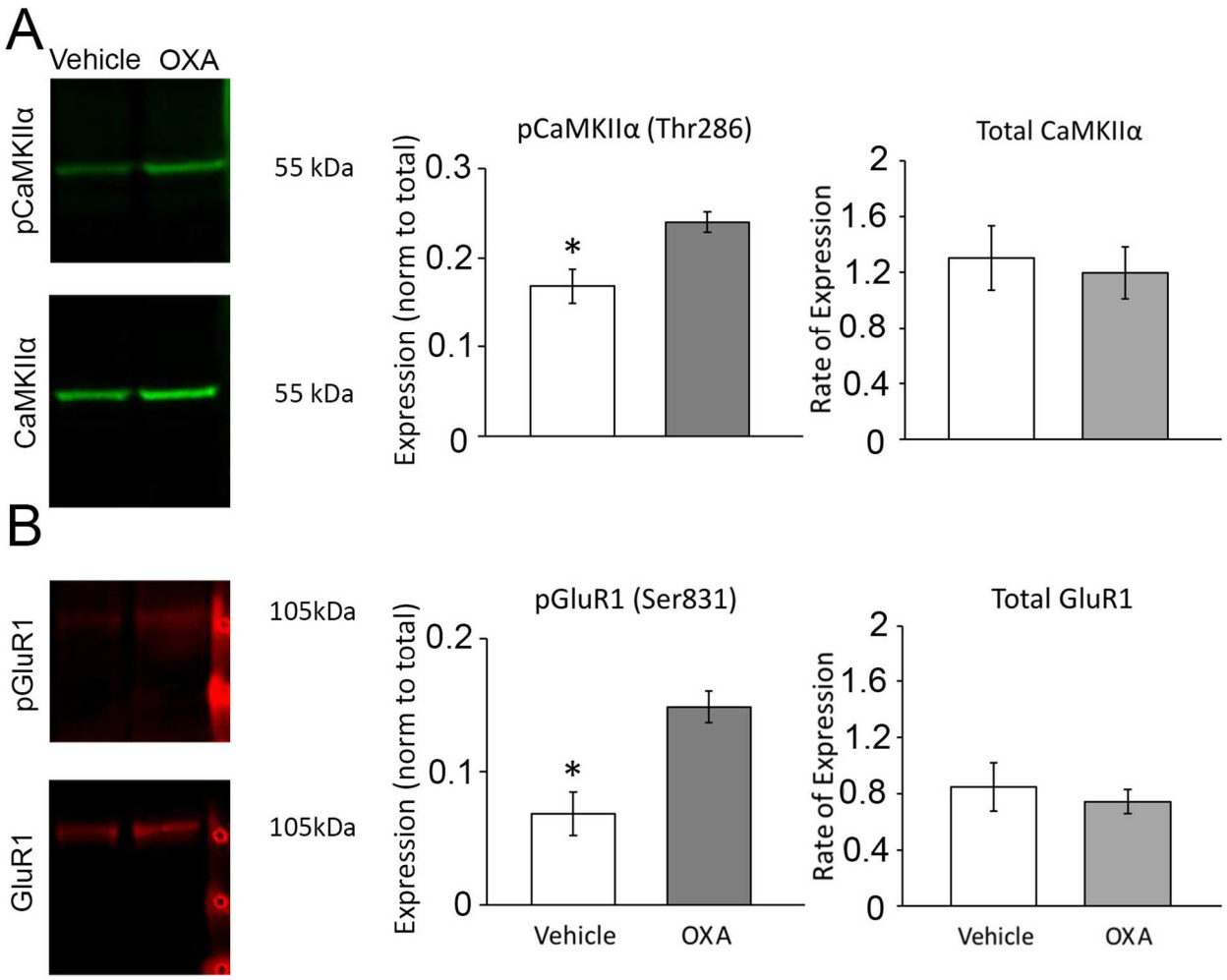

Figure 3.2 Intranasal OXA increases CaMKII and GluR1 phosphorylation when administered multiple times during training. (A) OXA-treated animals exhibit a higher relative ratio of phosphorylation for CaMKIIα(Thr286) while no significant differences were detected in total CaMKIIα expression when OXA is administrated during the acquisition phase of MWM training (n=8/condition; independent-samples *t*-test; CaMKIIα(Thr286)- $t(14)= -3.219$; $p= 0.006$; CaMKIIα- $t(14)= 0.358$; $p= 0.725$). (B) Phosphorylation of GluR1 (Ser831) was significantly higher in OXA-treated animals while no significant differences were detected in total GluR1 expression in either group (GluR1(Ser831)- $t(14)= -3.984$; $p= 0.001$; GluR1- $t(14)= -1.089$; $p= 0.295$). Data are represented as mean ± SEM. *$p<0.01$.

Long-term Knockdown of OX1Rs in the dHPC Impairs MWM Performance

We verified our stereotaxic coordinates to ensure accurate placement of the viral knockdown and transfection within the dHPC (Figure 3.3A). Additionally, we ran Western Blots on an additional cohort of animals to verify the rate of OX1R knockdown of the vector. Hippocampi that were injected with OX1R-shRNA exhibited approximately a 40% knockdown in OX1R expression when compared to scrambled(sc)-shRNA controls (See Appendix B, Figure S3.4A and S3.4B; independent-samples *t*-test; $t(10)= 5.338$, $p<0.001$).

Once MWM training and testing finalized, a mixed-model ANOVA revealed a significant interaction between training days and rAAV treatment when assessing the first training trial (24h delay) across the training period (Figure 3.3B; mixed model ANOVA with simple main effects *post-hoc analysis*; interaction: $F(4,52)= 2.706$, $p<0.05$; simple main effects: Day 3- ($F(1,13)= 5.936$, $p<0.05$), Day 5- ($F(1,13)= 12.407$, $p<0.01$). When simple main effects were analyzed following the significant statistical interaction, animals injected with OX1R-shRNA displayed a higher latency in locating the platform on the third and fifth day of training. By the time training finalized, sc-shRNA animals could locate the platform approximately 60 seconds faster than their OX1R-shRNA counterparts in the long-term learning assessment. When short-term learning was assessed by comparing the second training trial throughout the training period (30s delay), a mixed-model ANOVA revealed a significant main effect of training across five days (Figure 3.3C; Main effect of treatment condition: $F(1,13)= 2.183$, $p>0.05$; interaction: $F(4,52)= 0.769$, $p>0.05$). Both the OX1R-shRNA and sc-shRNA cohorts demonstrated reduced latency in locating the platform by the time training finalized. Although there was no significant main effect of treatment condition, figure 3.3C shows a trend of the sc-shRNA group exhibiting a lower latency to locating the platform in the short-term learning assessment when compared to

the OX1R-shRNA. Control animals spent a higher amount of time in the goal quadrant compared to OX1R knockdowns (Figure 3.3D and 3.3E; one-tailed independent-samples *t*-test; $t(13)= 1.947$, $p<0.05$). Furthermore, the time controls spent searching for the platform in the goal quadrant was significantly higher than chance levels (15s; one-sample *t*-test; $t(6)= 4.907$, $p<0.01$) while OX1R knockdowns performed at chance level (one-sample *t*-test; $t(7)= 1.437$, $p>0.05$. No significant differences in swim speed ($t(13)= -0.323$, $p> 0.05$) nor thigmotaxic behavior ($t(13)= -1.885$, $p>0.05$) were observed in both groups.

OX1R Knockdown in the dHPC Impacts CA1 Synaptic Plasticity

Morphometric analyses revealed OX1R knockdown animals exhibited a decrease in mushroom-shaped dendritic spines within the CA1 when compared to control animals (Figure 3.4A and 3.4B; $t(10)= -5.555$, $p<0.001$). However, the number of thin and stubby spines were not significantly different between both groups (Figure 3.4A and 3.4B; Thin spines: $t(10)= 0.463$, $p>0.05$; Stubby spines: $t(10)= -1.307$, $p>0.05$). CA1 BDNF protein expression was greater for the sc-shRNA group when compared to the OX1R-shRNA group (Figure S3.4C and S3.4D). A Mann-Whitney U test revealed that CA1 BDNF protein expression was greater for the sc-shRNA group (mean rank= 8.5) when compared to the OX1R-shRNA group (mean rank= 4.5) ($U= -2.051$, $p<0.05$).

Discussion

In this study, we demonstrate the involvement of the orexinergic system in hippocampal function that is influenced by environmental lighting conditions in a diurnal animal model. Diurnal grass rats in chronic dim lighting conditions displayed improved water maze performance when OXA was administered intra-nasally multiple times while acquiring the task. However, a single administration of OXA following training did not significantly impact

performance to improve reference memory. Moreover, higher phosphorylation rates of CaMKIIα(Thr286) and GluR1(Ser831) were indicative of increased induction of early LTP (E-LTP) as a consequence of recurring IN OXA applications throughout the training phase. The lack of changes in phosphorylation rates in the single-administration paradigm mirrored the absence of significant differences in MWM performance, which may suggest that these specific post-translational modifications may be how OXA exerts its effect on spatial learning and memory. Conversely, knockdown of OX1Rs within the dHPC impairs water maze performance in grass rats that have never been subjected to chronic daytime light deficiency. Hippocampal OX1R knockdown also induced a decrease of hippocampal BDNF expression and mushroom-shaped dendritic spines that may suggest that maintenance of late LTP (L-LTP) is impeded. These results show that the orexinergic system has the capability to bi-directionally influence hippocampal function based on the animals' long-term ambient light conditions during housing. However, it is worth mentioning that the bi-directionality of orexin signaling is asymmetrical because of the nature of the interventions that will be discussed in greater detail.

A key finding in this study is the improvement of MWM performance in grass rats despite being in chronic dim lighting conditions for four weeks when OXA is administered intra-nasally during pre-training for five days. Although no significant differences were observed between controls and OX-A-treated animals when assessing the trials with a 24h delay, the robust improvement of the OXA group in trials with a 30s delay may be acting preferentially on the acquisition of the task. At first glance, these results may appear to be contradictory to our previously published studies of dim lighting reversely impacting the animals (i.e., effects detectable in the 24h delay and not in the 30s delay) [180]. However, a potential explanation for this occurrence might be that OXA might be re-strengthening the trace memory that has been

slowly developing throughout previous trials that might weaken over long delay periods. Since OXA may be exerting this effect over the entire course of training (i.e. five days), by the time OXA administrations cease to test reference memory, OXA-treated animals may still have a stronger memory trace than their vehicle counterparts despite possible weakening due to exogenous OXA no longer being administered.

Previous work has demonstrated that direct infusion of an OX1R antagonist pre-MWM training into the dentate gyrus of the dHPC impairs acquisition [76]. Because of the nature of IN administration, OXA has been demonstrated to remain elevated within the CNS at least 2h following the administration [220], therefore, IN OXA may also be acting upon the consolidation phase (post-training) within our paradigm. Because our MWM training paradigm consists of 10 total sessions (two/day), compensatory training mechanisms involving cortical brain regions may be masking some impairments brought on by decreased OXA [222]. This evidence could support why we observe an effect on the short-delay trials but not on long-delay ones, a training paradigm with fewer sessions could resolve this.

Since the administration method of OXA into the CNS was not limited to a region, multiple areas could be working in conjunction to prevent MWM impairments due to chronic daytime light deficiency. One could argue that because of its involvement in arousal [223], OXA might be yielding better MWM performance due to our animals displaying higher levels of wakefulness. While this may be a possibility, our lab has demonstrated that there are no significant differences in the amount of locomotor activity during the LD cycle when housed in either brLD or dimLD for four weeks [47]. Additionally, due to the lack of differences in swim speed and thigmotaxic behavior from IN OXA, it is likely that the behavioral effects that we

have observed in this study are due to OXA's impact on cognitive function instead of other functions that are associated with this neuropeptide.

The increase of the ratio of autophosphorylation of CaMKIIα(Thr286) and CaMKIIα-mediated phosphorylation of GluR1(Ser831) within the HPC that we observed when OXA was administered pre-training provides molecular signaling evidence as to how learning and memory are affected. Robust elevation of Ca^{2+} through N-Methyl-D-aspartic acid receptors (NMDARs) triggers phosphorylation of CaMKIIα(Thr286), which then binds to NMDARs at the post-synaptic density (PSD) [224, 225] to traffic and phosphorylate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) such as GluR1 to enable induction of LTP at hippocampal synapses [226]. These molecular substrates could point towards OXA exerting an increase in basal synaptic transmission rates that trigger the secondary events mentioned that result previously in LTP. Through this mechanism, pre-training IN administration of OXA may enable cognitive priming of the HPC to incorporate location information gathered from the first training session to be utilized for the subsequent session just 30s later that translates into a lower latency to find the platform when compared to animals that do not receive OXA.

Some limitations in our approach include the use of a non-fluorescently tagged peptide for IN administration. By not utilizing a fluorescent peptide we are not able to visualize to which brain regions the exogenous OXA is reaching, although we do have evidence that exogenous OXA is indeed acting upon the CNS; animals administered with a 10nmol dose exhibit increased thigmotaxis when compared to animals given a 5nmol dose and controls. Previous evidence has demonstrated that excessive OXA may increase anxiety-like behavior [227, 228], which is what is observed when grass rats are administered with a high dose of OXA. Additionally, IN administration of OXA does not selectively target the HPC; instead it is globally distributed

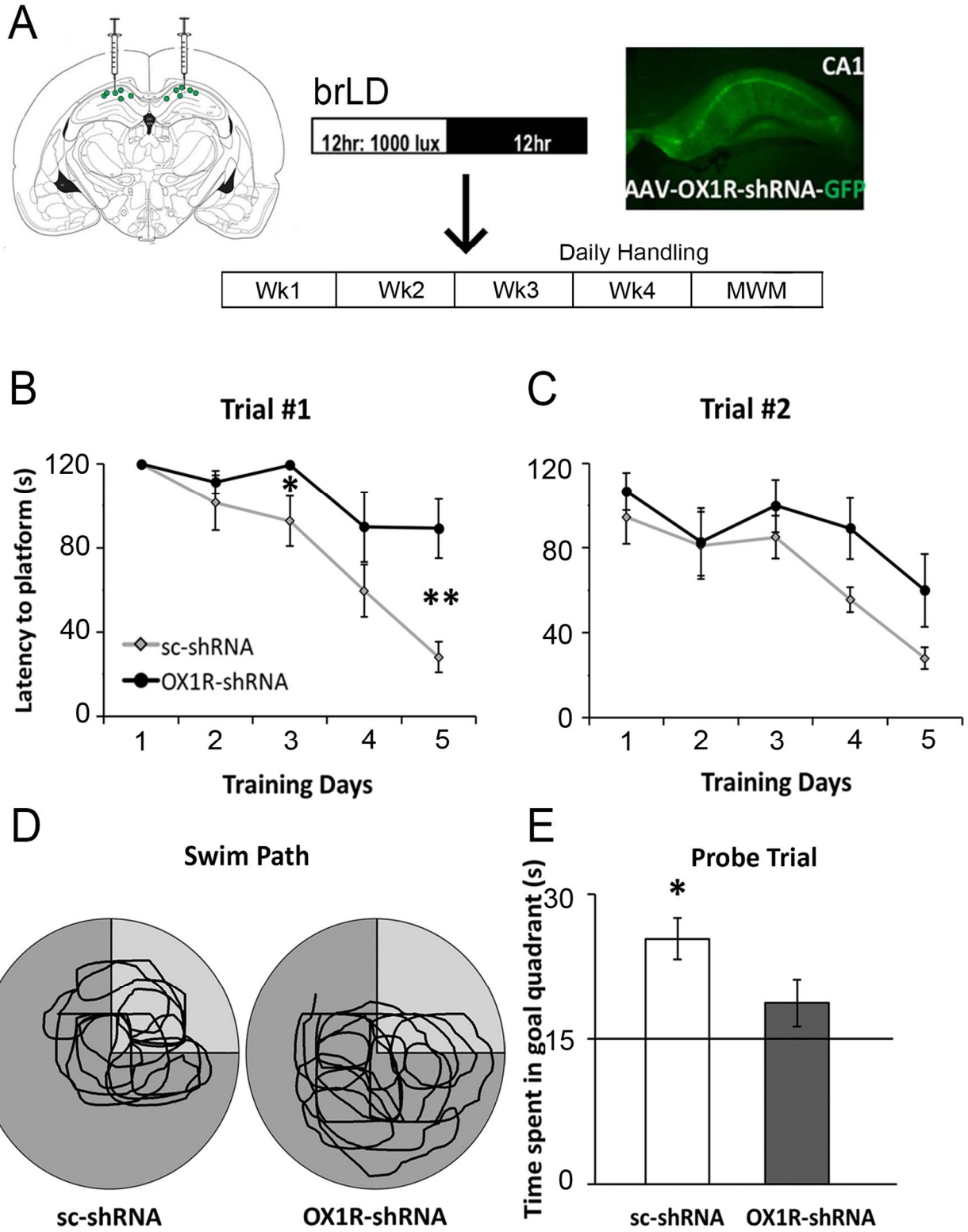

Figure 3.3 Knockdown of OX1Rs in the dHPC and behavioral outcomes in the MWM. (A) Representative photomicrograph of viral transfection of AAV-OX1R-shRNA within the CA1 of

Figure 3.3 Knockdown of OX1Rs in the dHPC and behavioral outcomes in the MWM (cont'd). the dHPC along with the lighting condition and timeline of the experiment. (B) Animals that were injected with rAAV1-OX1R-shRNA exhibited longer latencies to locate the platform during the first training trial throughout the training period (n=7-8/condition; mixed model ANOVA with simple main effects *post-hoc analysis*; interaction: $F(4,52)= 2.706$, $p<0.05$; simple main effects: Day 3- ($F(1,13)= 5.936$, $p<0.05$), Day 5- ($F(1,13)= 12.407$, $p<0.01$). (C) dHPC Although there was a trend of OX1R knockdown animals exhibiting longer latencies to locate the platform during the second training trial throughout the training period, these differences were not statistically significant (Main effect of treatment condition: $F(1,13)= 2.183$, $p>0.05$; interaction: $F(4,52)= 0.769$, $p>0.05$). (D) Representative swim paths for animals in both lighting conditions. (E) OX1R knockdown animals spent less time searching for the platform in the goal quadrant (one-tailed independent-samples *t*-test; $t(13)= 1.947$, $p<0.05$); control animals searched in the goal quadrant higher than chance level (one-sample *t*-test; $t(6)= 4.907$, $p<0.01$) while knockdown animals did not differ from chance ($t(7)= 1.437$, $p>0.05$).

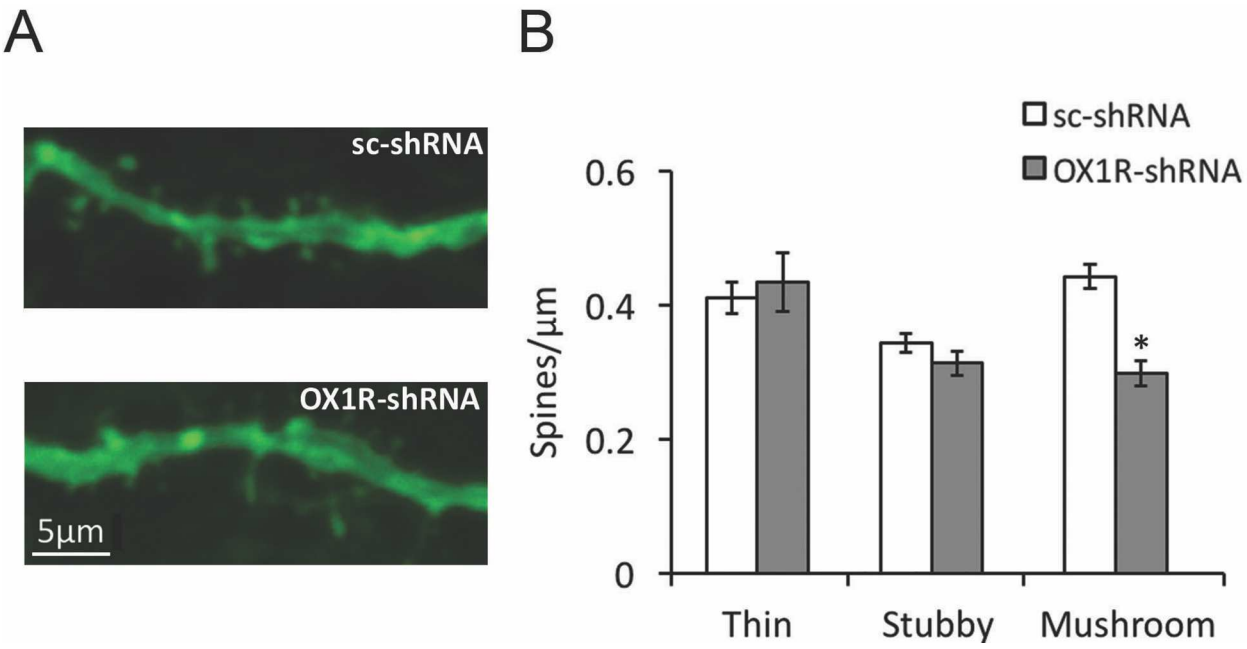

Figure 3.4 OX1R knockdown in the dHPC impacts CA1 dendritic spine density. (A) Representative photomicrographs of dendritic segments in the CA1 of animals introduced with the knockdown or scrambled control vectors. (B) OX1R knockdown animals exhibited a decrease in mushroom-shaped dendritic spines within the CA1 when compared to control animals but not in thin or stubby spines (n=6/condition; independent-samples *t*-test; Mushroom- $t(10)= -5.555$, $p<0.001$; Thin- $t(10)= 0.463$, $p>0.05$; Stubby- $t(10)= -1.307$, $p>0.05$). Data are represented as mean ± SEM. *p<0.001.

across the CNS. This non-specific distribution may result in OXA acting upon other brain regions such as the retrosplenial cortex (RSC) and the prefrontal cortex (PFC) that play significant roles in the store long-term storage and retrieval of spatial memory, respectively [229,

230]. Another limitation that is present within the design is the lack of inclusion of two (one OXA-treated and the other vehicle-treated) animal groups housed in brLD conditions, we were limited in the availability of subjects to carry out a design with four groups while achieving appropriate statistical power. The inclusion of these two groups would allow for a more complete interpretation based on how these animals perform. We would anticipate vehicle-treated brLD grass rats would exhibit a similar learning curve as our previous study [180], while there would be a possibility that OXA-treated animals may perform worse due to excess OXA confounding behavioral results due to an increase in thigmotaxic behavior as observed in animals given 10nmol of OXA.

Overall, our results suggest that OXA may be able to prevent spatial learning and memory impairments induced by chronic daytime light deficiency within a diurnal animal model. However, further testing paradigms need to be conducted before confirming this statement. The behavioral effects are achieved, in part, via phosphorylation of CaMKIIα and GluR1 to elicit the induction of hippocampal LTP. Moreover, the behavioral outcomes seen when OXA is administered before acquiring the task seem to carry over for long-term memory retrieval. The same cannot be said for when OXA is given after the task has been acquired, which confines OXA actions on learning and memory to the acquisition and consolidation phases.

Grass rats subjected to viral vector-mediated knockdown of CA1 OX1Rs demonstrated a reduced learning curve during MWM training when compared to controls that were administered a vector containing scrambled shRNA. Additionally, OX1R knockdown animals did not retain the location of the platform as well as controls during the probe test for reference memory. Unlike the IN experiment, long-term knockdown of CA1 OX1Rs seems to affect performance in

the first trial (24h delay) instead of the second trial (30s delay). This occurrence may be due to the alteration of the orexinergic signaling pathway taking place across a more extended period which may affect HPC-dependent learning and memory performance through a distinct mechanism or, more likely, influencing a different phase of LTP altogether.

Another key difference that was observed in this experiment was that a stabilized, long-term knockdown of CA1 OX1Rs also produced a reduction of BDNF expression that was not observed in the previous experiment. Attenuation of BDNF expression may be due to the long-term reduction of OX1R signaling influencing specific kinases such as protein kinase C (PKC) and phosphoinositide 3-kinase (PI_3K) [231]. Moreover, viral vector-mediated deletion of postsynaptic BDNF within the CA1 has shown to impair LTP maintenance while induction of LTP remained intact [232]. This may explain the behavioral outcomes of OX1R-shRNA animals exhibiting impaired latencies in the first training trial and average latencies in the second trial.

In line with previous observations within our chronic daytime light deficiency paradigm, CA1 OX1R knockdown resulted in the reduction of mushroom-shaped dendritic spines [180]. Given that long-term learning and memory was affected while short-term learning and memory remained largely unaffected in the MWM, the anatomical findings of only mushroom dendritic spines being affected by OX1R knockdown correlate with postsynaptic BDNF being impaired. Hippocampal BDNF downregulation has shown to decrease the head width of dendritic spines in mature neurons by reducing F-actin levels that are crucial for dendritic architecture [233]. A possible explanation of these results may be that knockdown of CA1 OX1Rs selectively impact long-term memory through its actions on BDNF, which directly affect scaffolding proteins within dendritic spines to decrease head width rather than induce retraction of dendritic spines.

The current study proposed that the orexinergic system is a potential mediator for light-modulated hippocampal function in diurnal grass rats. By providing OXA in lighting conditions where OXA is depleted, we managed to improve performance impairments in the MWM when administered during acquisition of the task which demonstrated that orexin was sufficient in restoring hippocampal function. However, CA1-specific genetic knockdown of OX1Rs resulted in MWM deficits despite animals being in lighting conditions that increase orexin expression, which demonstrates that orexin is necessary for light-modulated hippocampal function. By uncovering some of the neural mechanisms of how light impacts cognitive function, further studies can be conducted to examine the viability of the orexinergic system as a potential treatment option in conditions where cognitive decline is a hallmark or comorbid symptom.

CHAPTER 5:

Conclusions

Framework

Research in biological rhythms has historically identified the LD cycle as the most crucial environmental component for the entrainment of circadian rhythms in various organisms including humans [234]. Additionally, light has been shown to influence several functional aspects within mammalian organisms that range from involuntary physiological functions [2] to affective state and cognitive function [3, 42]. Light-related disorders such as SAD and glaucoma have been well-identified in presenting comorbid symptoms of impaired cognitive function [21, 22, 244]. The benefits of bright light on cognitive function seem to extend to both patients of neurodegenerative diseases and healthy populations [26, 27, 104, 107, 146, 211]. Although fMRI studies have made several advancements by revealing that different wavelengths of light can elicit excitatory responses in various brain regions in humans, the exact neural mechanisms of how lighting conditions impact cognitive function were still unknown. Primarily, this gap in scientific knowledge is due to the lack of an appropriate animal model to study the effects of light on cognition.

The most commonly utilized mammalian animal models for research in the life sciences are domesticated/inbred strains of mice (*Mus musculus*) and rats (*Rattus norvegicus*). These traditional models have been used extensively due to physiological similarities with humans, and because of their lower cost for maintenance and breeding. A limitation for these animals is their nocturnal activity pattern being opposite from the diurnal pattern exhibited by humans. Considering many biological processes display circadian rhythmicity and that an organism's chronotype (diurnal vs. nocturnal) dictates differing physiological states in both the brain and behavioral measures during the day and night [31], a diurnal animal model would be more suitable to study the neural mechanisms of how light influences cognition.

Overall Approach

The work presented in this dissertation used the Nile grass rat as a diurnal animal model to investigate the mechanisms underlying light-modulated hippocampal function. The studies that I have conducted throughout the chapters is innovative not only because of the use of a diurnal rodent as my model, but also marks the first instance of administration of pharmacological compounds via the intranasal route and viral vector-mediated knockdown of a specific target have been done using grass rats. Additionally, I have also implemented a multi-tier level approach to gain insight into how light impacts hippocampal-dependent learning and memory. These approaches include behavioral assays, morphometric analyses, histology, protein expression, and molecular signaling.

Chronic Daytime Light Deficiency and its Effect on the Hippocampus

In Chapter 2, I implement the chronic daytime light deficiency paradigm that our lab has previously established to replicate seasonal variations of light intensity within laboratory conditions [47, 102, 103] to assess HPC-dependent learning and memory. Following four weeks of exposure to dimLD conditions, grass rats exhibited impaired MWM performance when compared to their brLD counterparts. During training, deficits were most noticeable when the inter-trial interval was extended to 24 hours as opposed to 30 seconds, where no significant differences were observed. These impairments persisted once training finalized and reference memory was tested. In a subsequent experiment to determine if these behavioral deficits were permanent, it was revealed, in a separate cohort of animals, that exposure to brLD conditions for an equal amount of time following dimLD exposure was sufficient to rescue these behavioral deficits.

Immunohistochemical assays revealed that hippocampal BDNF expression was attenuated following exposure to dimLD conditions, most notably within the CA1 subregion of the dHPC. However, mirroring behavior, exposure to the "therapy paradigm" was able upregulate BDNF expression. Because of these behavioral and immunohistochemical observations, we predicted that the morphology of hippocampal dendritic spines was also going to be negatively impacted. This was based on hippocampal BDNF demonstrating to have a functional role in synaptic plasticity by serving as a catalyst for LTP and by regulating downstream factors that influence dendritic spine dynamics to support learning and memory [100, 101, 154]. Consequentially, dimLD conditions decreased hippocampal dendritic spine density with the CA1 subregion being most affected, and the therapy paradigm spurred a re-emergence of dendritic spines. A more in-depth morphometric analysis revealed that mushroom spine subtypes, characterized by enhanced glutamatergic signaling crucial to long-term memory, were those that were most affected by chronic daytime light deficiency. Taken together, these results show that environmental lighting conditions have the capacity to induce functional and structural changes that extend beyond light's immediate effect on arousal to influence cognition. Lastly, the work done in this chapter has served as a foundational basis to uncover the signaling pathways that are responsible for mediating ambient light's effect on HPC-dependent learning and memory.

Future studies that would provide deeper insight into how environmental lighting conditions impact hippocampal function, would be to assess MWM performance in both brLD and dimLD conditions using a shorter training protocol. The current protocol implemented is a five-day, 10-session paradigm that yields significant differences during trials where the delay is 24h but not in those where the delay is 30s. A 2-day, 4-session protocol may result in more

drastic differences in throughout the training phase as well as the testing day. A previous study demonstrated that application of an OX1R antagonist negatively impacted MWM performance when animals were subjected to weak training (only two training sessions), but not strong training (six training sessions) [222]. In the chronic daytime light deficiency paradigm, in which grass rats exhibit reduced OXA expression, extensive training may ameliorate deficits by compensating with the activation of other brain regions essential in spatial memory like the RSC and the subiculum. Therefore, a shorter training phase may be more dependent upon OXA's influence in the HPC.

To expand on the findings from Chapter 2, the experiments done in Chapter 3 were aimed towards investigating the effects of ambient lighting upon HPC-dependent learning and memory in female grass rats, given that the experiments in Chapter 2 were carried out in males. Because certain conditions such as SAD, Alzheimer's Disease and dementia have a substantially higher prevalence rate in women when compared to men [192, 212, 235], it is of interest to replicate the experimental paradigm to assess how environmental lighting conditions impact the female population in our model and identify any potential sex differences. As in Chapter 2, female grass rats exposed to dimLD conditions exhibited impaired MWM performance when compared to animals housed in brLD. However, one interesting finding that was observed was that throughout the training period, female grass rats in dimLD did not exhibit a learning curve as training days progressed. In contrast, males in dimLD conditions demonstrated a learning curve during training, albeit a slower curve in comparison to brLD. The differences in behavioral output between both sexes seem to suggest that ambient lighting seems to have a more pronounced effect in female grass rats, which mirrors the higher rate of susceptibility that women possess in being diagnosed with dementia and depressive disorders.

One finding that was consistent between male and female grass rats was that dimLD conditions negatively impacted hippocampal dendritic spine density within the CA1 of the HPC. Mushroom spine subtypes were the ones that were affected at the highest rate in females as well. However, unlike males, females did not exhibit any significant differences in hippocampal BDNF expression. Before suggesting that lighting conditions affect a distinct signaling pathway in females, ligand-mediated phosphorylation of BDNF's receptor, TrkB, was assessed. Out of the three main signaling cascades that BDNF-TrkB binding interactions possess, the PLCγ–$Ca2^{+}$ pathway is prevalent at CA3-CA1 synapses to aid in LTP induction [98]. Western Blotting procedures to determine the expression of the TrkB(Tyr816) phosphoprotein did not reveal any significant differences in females in either lighting condition. Although many studies indicate that there is a relationship between sex hormones and BDNF-TrkB signaling, the effects of steroid signaling onto the BDNF-TrkB pathway is still unclear. Moreover, because female grass rats have a sex hormone profile resembling that of induced ovulators, cycling levels of hormones in adulthood may not be the primary reason for the observed sex differences. Alternatively, rodent and human studies have shown that females and males use different search strategies within the water maze [181, 236]. This posits the argument that because of the differences of search strategy, the molecular signaling mechanisms might be completely different.

To improve the quality of the work presented in this chapter, an experiment worth carrying out to determine which molecules are negatively impacted by chronic dim lighting conditions in female grass rats would be to run single-cell mass spectrometry [245]. This technique allows for the chemical profiling in single living neurons to detect changes in small, intracellular molecules during physiological processes. Specifically, by combining patch clamp techniques with induced nano mass spectrometry, it is possible to survey hundreds of molecules

while maintaining cell viability to record neural activity as well. While it would be difficult to predict which molecule(s) would be involved as downstream targets of light-modulated hippocampal function in female grass rats, two potential candidates could be NF-κB and p25. NF-κB and p25 have been shown to play a more significant role in hippocampal LTP and compensation of memory deficits in Alzheimer's Disease, respectively [209, 241] within female rodent models. Because our lab has only gathered data on chronic daytime light deficiency negatively impacting OXA expression in male grass rats, it would also be of great interest to know how orexin responds in female grass rats.

Orexin is Essential for Light-Modulated Hippocampal Function

To make progress in identifying the neural mechanisms that mediate light's effect of HPC-dependent learning and memory, examining a direct retino-recipent area of the brain, such as the LH [237, 238], would be a primary focal point. Previous efforts found that orexinergic cells within the LH respond well to the LD cycle, with many of them being active during the light phase [132]. Additionally, we have found that our lab's established chronic daytime light deficiency paradigm attenuates OXA expression [103]. Given OXA and OX1R's involvement in HPC-dependent learning and memory [75, 76, 217], the experiments in Chapter 4 sought out to determine if manipulations of the LH-HPC orexinergic signaling pathway would alter light-modulated MWM performance.

The first experiment of that chapter examined the effects IN administration of exogenous OXA to grass rats exposed to dimLD conditions. The results of this experiment confirmed that by maintaining OXA levels at an optimal level, even when animals are exposed to chronic daytime light deficiency, this is ***sufficient*** to support hippocampal function. Additionally, molecular findings demonstrate that OXA can recruit essential mechanisms for the induction of

hippocampal LTP. IN administration of OXA resulted in an increase of CaMKIIα autophosphorylation and CaMKII-mediated phosphorylation of GluR1s to enhance glutamatergic signaling vital for LTP induction.

With the use of recombinant viral vectors to generate a permanent knockdown of OX1Rs within the CA1 of the HPC in grass rats, I wanted to test the hypothesis of the dampening of orexin signaling within the HPC would lead to impairments in MWM performance as previously observed. Once the experiment was finalized, the results confirmed the established hypothesis that knockdown of hippocampal OX1Rs would induce a similar behavioral phenotype as when animals were subjected to dimLD conditions. Furthermore, knockdown of hippocampal OX1Rs also resulted reduced BDNF expression and mushroom dendritic spine density that have been established as substrates key to synaptic plasticity. The interesting detail of this finding that is worth mentioning is that these animals were housed in brLD conditions and the specific disruption of oxinergic signaling confirms that the orexinergic system is ***necessary*** in ambient light's influence on hippocampal function.

To further investigate the orexinergic system's role in mediating light-modulated hippocampal function, a series of studies in which distinct, direct manipulations from the ones previously discussed could provide a further, detailed understanding. For one, another limitation of the intranasal experiments that has not been mentioned is the lack of clarification at what phase of the learning and memory process does OXA exert its effect to improve HPC-dependent memory. A suitable approach to tackle this question, would to incorporate a chemogenetic approach known as designer receptors exclusively activated by designer drugs (DREADDs). DREADDs are viral vectors that are specifically designed to either promote or inhibit neural activity within a specific neuronal population [246]. Rather than becoming functional active once

incorporated into the animal's genome, it only becomes temporarily active upon the administration of clozapine-N-oxide (CNO), a synthetic ligand that activates DREADDs with relatively little pharmacological impact elsewhere. Because CNO has faster wash out rate when compared to IN OXA (40-70 min. vs. 120+min., respectively), this approach would allow a better controlled timescale to decipher orexin's specific phase involvement in HPC-dependent learning and memory. By incorporating an hM3Dq DREADD into the lateral hypothalamus, orexinergic neurons can be specifically activated before or after training sessions to elucidate their involvement in acquisition and consolidation, respectively; activation prior to the probe test would allow to determine if orexin is involved in retrieval. Furthermore, another study that can be undertaken is to utilize AAVs to selective upregulate dHPC OX1Rs and evaluate MWM performance to see if improvements occur despite being exposed to dimLD conditions. With this approach, it could be determined if enhancing and dampening the OXA-OX1R signaling pathway from the lateral hypothalamus to the dHPC can produce a symmetrical, bi-modulatory effect.

Impact

Overall, the work presented here has developed a paradigm within an adequate animal model to highlight the extent of how environmental lighting conditions can affect one crucial aspect of cognitive function: HPC-dependent learning and memory. The experiments in this dissertation have revealed that the associated neurobiological targets of ambient light's influence on cognitive processes within the HPC and show that the deleterious effects of dim lighting are not permanent. They also demonstrate sex differences in the grass rat, where females seem to be impacted to a higher degree when compared to males through seemingly distinct neural mechanisms. These findings may help shed some insight as to why there seems to be a higher

predisposition towards women suffering from certain conditions, like dementia and SAD, where environmental lighting conditions play a role in the occurrence of the condition and serve as a beneficial treatment option.

By identifying and showcasing the orexinergic system as the primary mechanism responsible for light-modulated hippocampal function, future efforts can be made into developing novel therapeutic strategies to combat the cognitive decline in dementia and depressive disorders. Suvorexant (aka.Belsomra®), an orexin receptor antagonist marketed for the treatment of insomnia, has demonstrated to impact learning and memory within humans by listing amnesia and "memory loss" as part of the side effects when taking the medication [239, 240]. This evidence highlights the importance of orexin in learning and memory, and this neurotransmitter system should be taken into consideration for pharmaceutical companies to develop potential products in their pipeline targeted at conditions where cognitive decline/impairment is a hallmark feature. Lastly, another potential impact that the work presented here may have in shaping public policy by raising awareness that the average American spends approximately 90% of their time indoors [44]. The dissemination of this information may spur infrastructure changes within schools, the workplace and homes to allow improved infiltration of natural sunlight in these spaces.

APPENDICES

APPENDIX A:

Chapter 2 Supplemental Figures

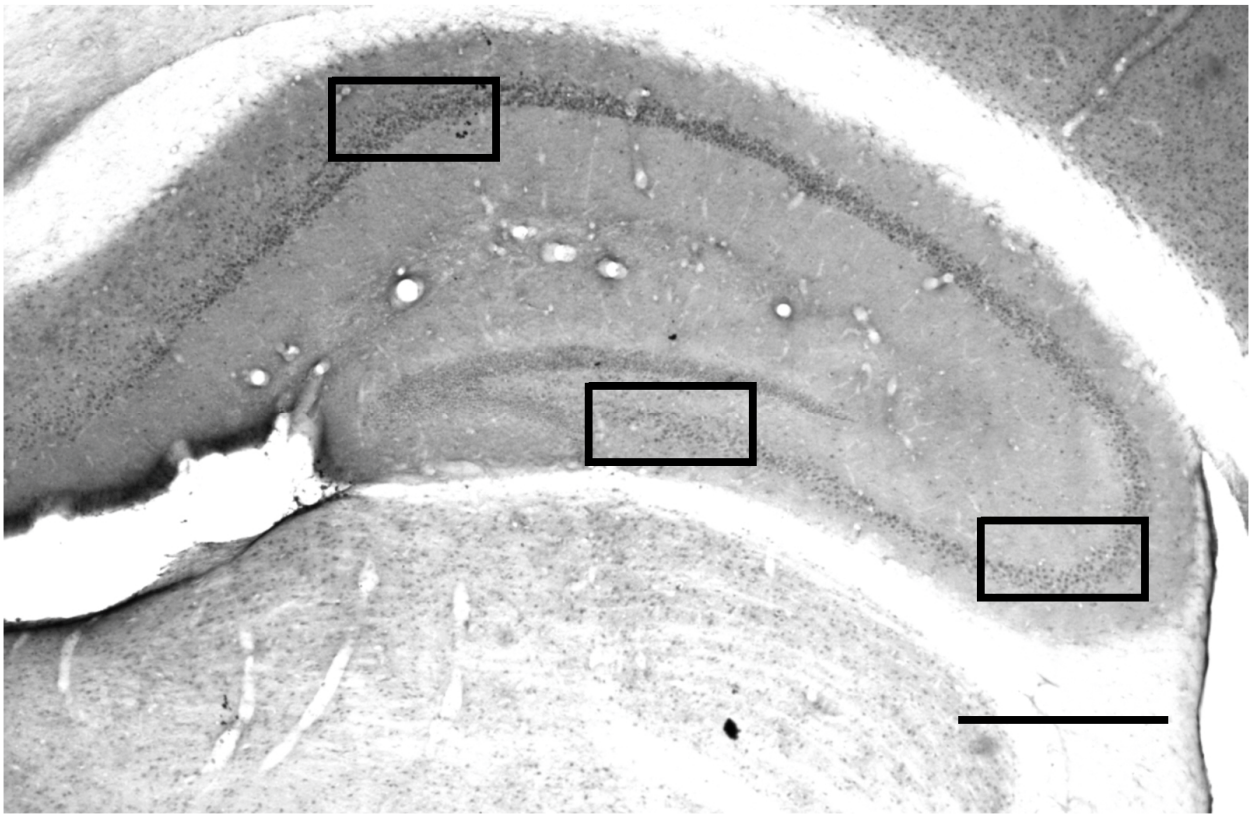

Figure S1.1 BDNF immunoreactivity within the HPC. Representative photomicrograph of BDNF-ir staining along with defined boxes that were used to quantify BDNF-ir cells in the CA1, CA3, DG regions. Scale bar, 400μm.

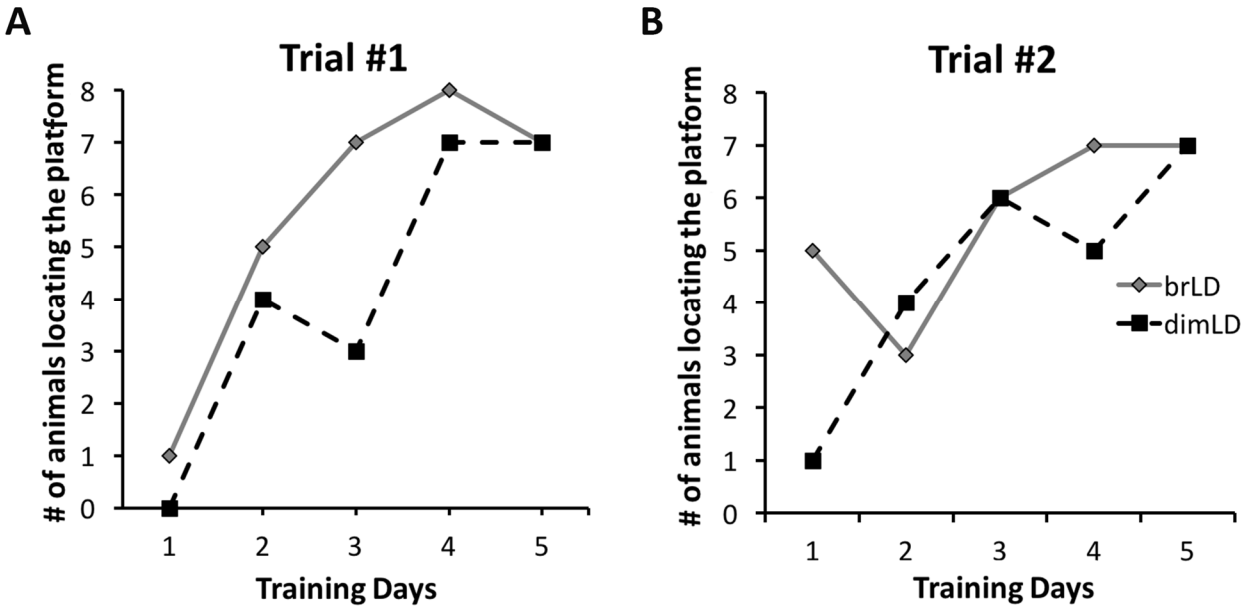

Figure S1.2 Successful localization of hidden platform during MWM training. (a) A higher percentage of grass rats in both lighting conditions can locate the hidden platform in the first training trial as the training period progresses. (b) In the second training trial, grass rats become more successful at locating the hidden platform as training finalizes.

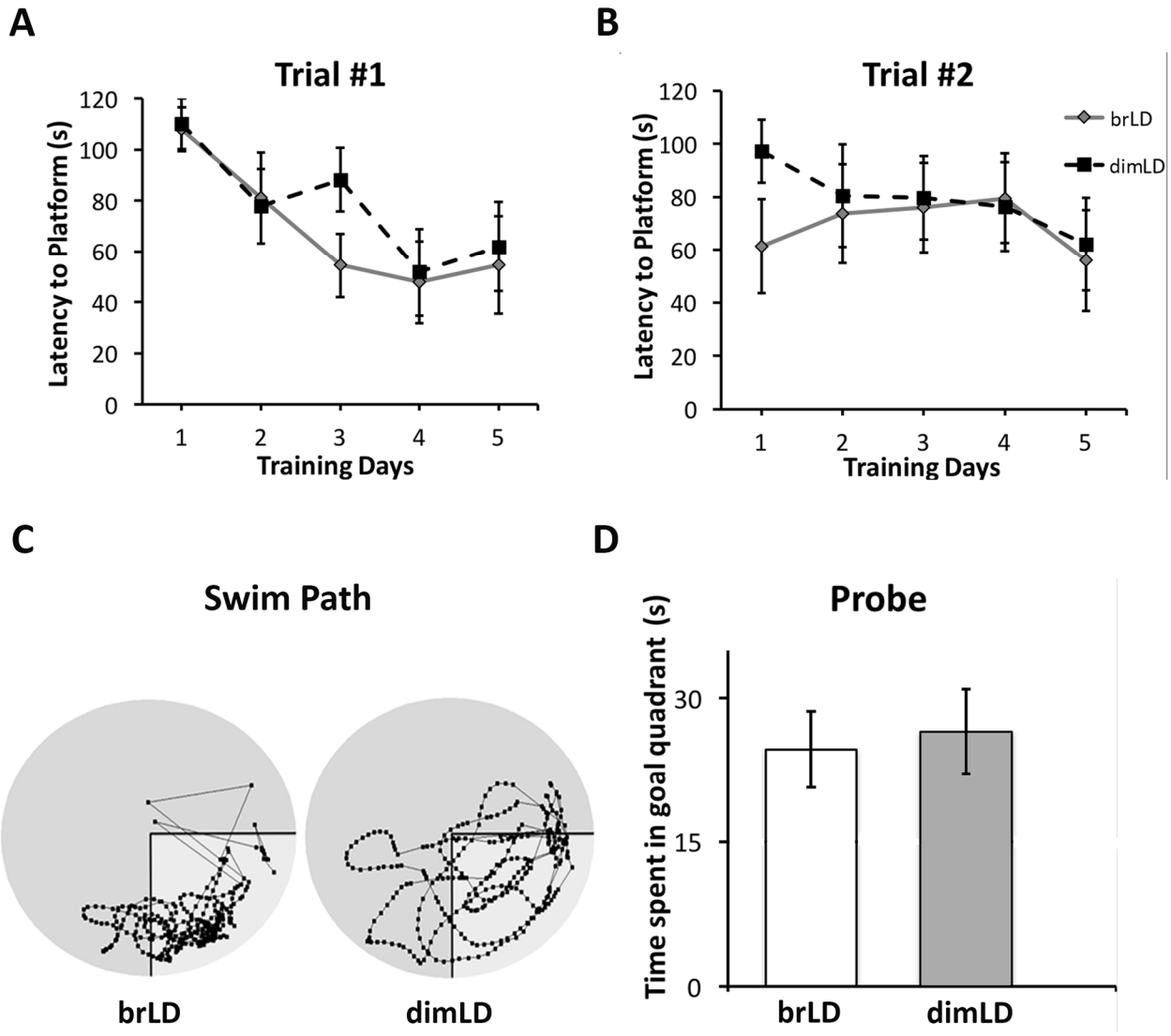

Figure S1.3 Short-term exposure to chronic daytime light deficiency does not negatively impact MWM performance. (a) Latency of animals to locate the platform during trial 1 (24 h delay) over the 5 training days. No significant differences were found between groups. (b) Latency of animals to locate the platform during trial 2 (30 s delay), there were no significant differences between the two groups. (c) Representative track plots of a grass rat in each lighting condition during the probe trial (with goal quadrant highlighted). (d) Grass rats in both lighting conditions spent about the same amount of time searching for the platform in the goal quadrant.

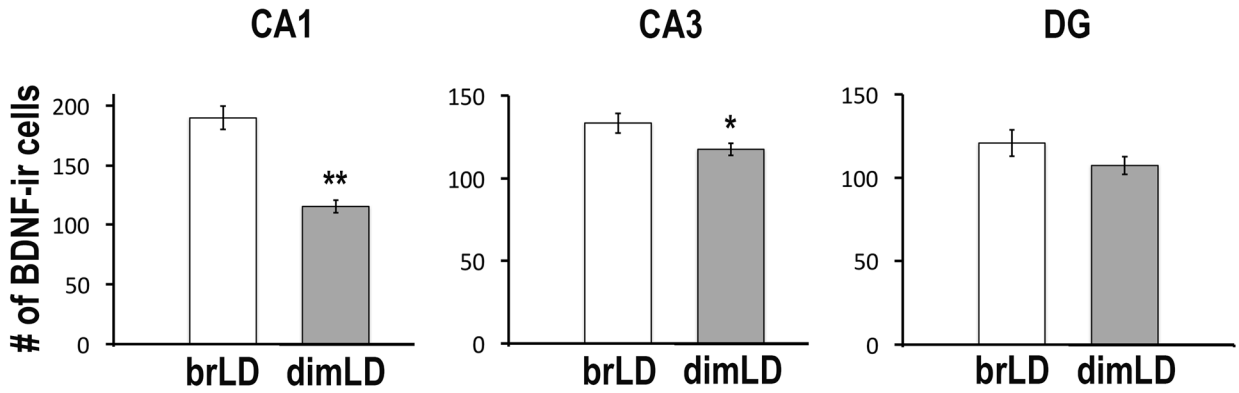

Figure S1.4 Ambient light condition modulates hippocampal BDNF expression. Number of BDNF-labeled cell bodies in each subregion in the hippocampus in behaviorally naïve animals housed in brLD or dimLD conditions. *, $p < 0.05$; **, $p < 0.001$

APPENDIX B:

Chapter 4 Supplemental Methods and Figures

SI Materials and Methods

Intranasal orexin A (OXA) peptide administration

In a separate experiment to assess the effects of IN OXA on retrieval aspects of the MWM task, animals in dimLD conditions (n=8/treatment) underwent IN administration 90 minutes prior to the retention probe trial, no IN administration occurred during the training phase. Once testing finalized, brains were collected and processed for Western blotting.

Western Blot

Flash frozen brains were sectioned coronally at 200 μm thickness, thaw-mounted onto a subbed slide. The CA1 subregions were punched out from the slice using a 1-mm (diameter) micropuncher (Harris Micropunch, Hatfield, PA) and stored at -80 °C. The tissue punches were then homogenized in radio-immunoprecipitation assay (RIPA) lysis buffer (sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA) containing phosphatase inhibitors (PhosSTOP™, Millipore Sigma) for protein extraction according manufacturer's instruction. Protein concentrations were measured with the Bradford assay method (Bio-Rad; Hercules, CA). 20 μg total protein was run on precast gels for all proteins of interest (4-20% Tris-Glycine Mini; Invitrogen cat#XP04200BOX) and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks; Invitrogen by Thermo Fisher Scientific). Membranes were treated with REVERT Total Protein Stain Kit (P/N 926-11016; LI-COR, Lincoln, NE) to quantify total protein for western blot normalization using the LI-COR Odyssey CLx Imaging System. After total protein imaging, membranes were washed with REVERT reversal solution to remove total protein stain and the membranes were proceeded for immunoblotting. All membranes were incubated in their respective primary antibodies for 72 hours at 4°C and in their secondary antibodies for 1.5 hours at room temperature.

For knockdown validation of OX1R in dCA1, behaviorally naïve grass rats were utilized to assess OX1R and mBDNF expression (n=6/condition). For OX1R, membranes were incubated in a rabbit anti-hypocretin receptor 1 primary antibody (1:1000, Abnova, cat# PAB8017). Membranes were incubated with a IRDye® 680RD goat anti-rabbit IgG (1:10000, 0.1mg, LI-COR, cat#925-68071).

Viral vectors

19 nt long siRNAs were designed against the grass rat's orexin receptor 1 (OX1R) cDNA sequence using standard algorithms as described previously. Candidates were blasted against available genome databases to ensure target specificity [242]. siRNAs in the form of shRNAs (loop TTCAAGAGA) were cloned into a rAAV genome behind a H1 promoter. The same genome also contained a separate GFP cassette as a transduction marker. Candidate shRNAs were tested using a luciferase reporter assay *in vitro*, and the shRNA with the highest *in vitro* efficacy (5' CCAACTACTTCATTGTCAA 3'; representing nucleotides 807- 825 of the OX1R) were selected for AAV production. A scrambled shRNA (5' CACAAGATGAAGAGCACCA3') was used as control. shRNA genomes were packaged into AAV2/9 using co-transfection of HEK293T cells of the rAAV genome, AAV9 rep/cap, and AAV helper functions. AAV particles were collected from lysed cells and media 72 hours later and purified using an iodixanol step gradient as described previously [243]. Titers were determined using dot blot (1.6×10^{13} vector genomes/ml (vg/ml; OX1R) and 1.4×10^{13} vg/ml (SCR)). Two vectors were produced to either knockdown OX1Rs (AAV9-hsyn1-OX1R-shRNA-GFP) or be used as a control for adequate comparisons (AAV9-hsyn1-SCR-shRNA-GFP).

Stereotaxic surgical procedure

Grass rats were anesthetized with 5% isofluorane during 5 minutes of induction period followed by 2.5% isofluorane throughout the remainder of the procedure using a low-flow anesthesia system (Somnosuite®, Kent Scientific). Neuros syringes (33-gauge; Hamilton; Cat#65460-03) were utilized to perform microinjections, all syringes were prepared with a siliconizing reagent (Sigmacote®, Millipore Sigma) to prevent adhesion of viral particles to glass surfaces. Once the entire volume of the viral vector was injected into the dCA1, the syringe stayed in place for five minutes prior to extraction to maintain viral spread localized to the target region.

MWM

A circular pool (60 cm depth x 122 cm diameter) was utilized with a platform (15 cm diameter) located 2 cm under the water level and approximately 30 cm away from the perimeter of the pool. The water was made opaque by adding white, non-toxic tempera paint and was kept at 26±2 ^{o}C. Different geometrical cues were posted up on each wall in the room for spatial orientation. A ceiling-mounted video camera was placed directly above the pool to record all sessions. Prior to training, animals were handled daily for seven days for five minutes to habituate them the experimenter. Then, a single cued training trial, in which the platform was visible, was introduced to assure that animals possessed typical motor abilities and recognized the platform as the avenue to solve the MWM [123]. Hidden-platform training took place across five days during daytime between 12:00-14:00 (5-7 hours after light onset), lighting intensity in the test room was ~300 lux. Each training day had two 2-minute sessions with a 30-second inter-trial interval. Every grass rat was placed in a different quadrant every trial in a pseudorandom order, so that no animal was placed in the same quadrant for two consecutive trials. 24 hours after the last training trial, the hidden platform was removed from the pool to assess reference memory in a 60-second probe trial. Hidden platform training and probe trial sessions were

recorded and analyzed using Noldus *Ethovision* program (*XT* 8.5, Noldus Information Technology, Netherlands) by an experimenter who was blind to the experimental conditions. During the training trials, latency to reach the platform was measured. Animals that failed to locate the platform at the end of the training trial were guided towards the platform and assigned a latency score of 120 seconds. During the probe trial, reference memory was assessed by quantifying the amount of time spent in the goal quadrant where the platform was located during training. Swimming ability was measured by assessing swim speed in all groups using the center point tracking option available on *Ethovision*.

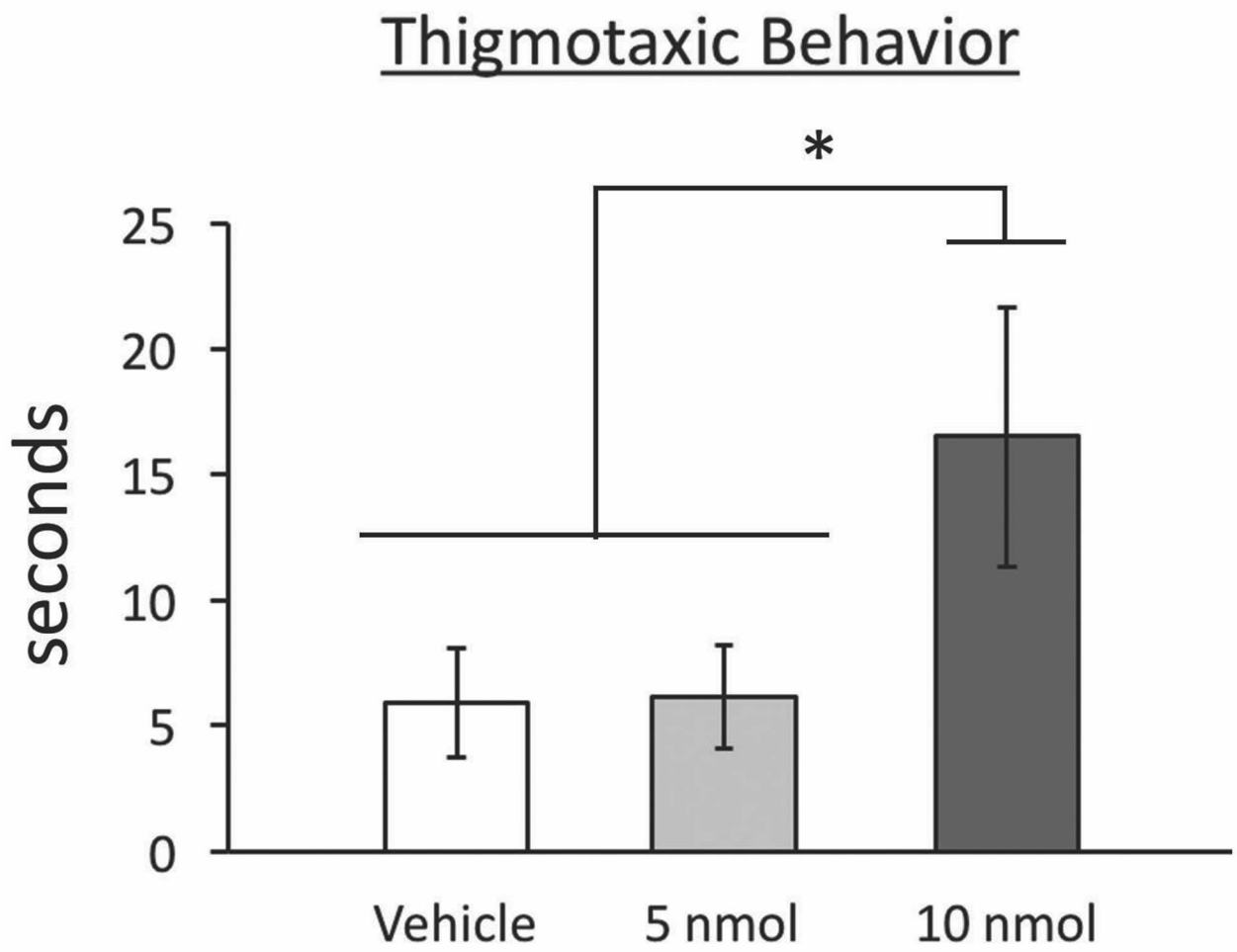

Figure S3.1 Doses of intranasal of OXA and thigmotaxic behavior. Animals that were administered with 10nmol of OXA exhibited higher rates of thigmotaxic behavior when compared to vehicle-treated animals or those treated with 5 nmol of OX-A (n=6/condition; one-way ANOVA: $F(2,17)= 21.022$, $p<0.001$; Games-Howell *post hoc* comparison: 10nmol vs. vehicle and 5nmol, $p<0.05$). Data are represented as mean ± SEM.

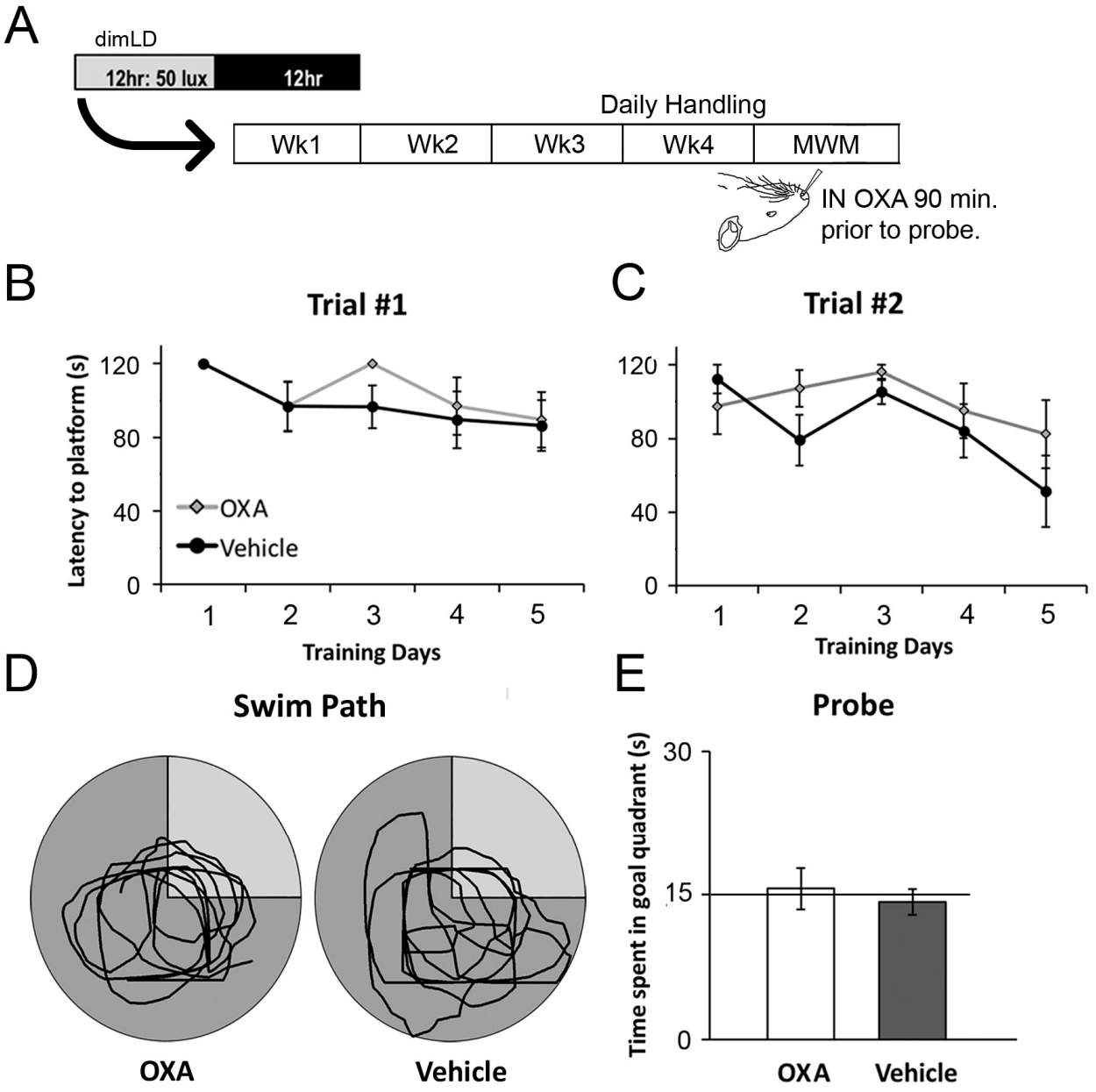

Figure S3.2 Single intranasal administration of OXA following training of MWM. (A) Four-week lighting paradigm along with the experimental timeline. (B) No significant differences emerged between both groups' latencies to locate the platform during the first training trial throughout the training period (n=8/condition; mixed model ANOVA; Main effect of treatment condition: $F(1,14)=0.417$, $p>0.05$; interaction: $F(4,56)=0.427$, $p>0.05$). (C) No significant differences emerged between both groups' latencies to locate the platform during the second training trial throughout the training period (main effect of treatment condition: $F(1,14)=1.332$, $p>0.05$; interaction: $F(4,56)=1.193$, $p>0.05$). (D) Representative swim paths for animals in both lighting conditions. (E) There were no notable differences in the amount of time spent searching for the platform in the goal quadrant (independent-samples t-test; $t(14)=0.238$, $p<0.05$). Data are represented as mean ± SEM.

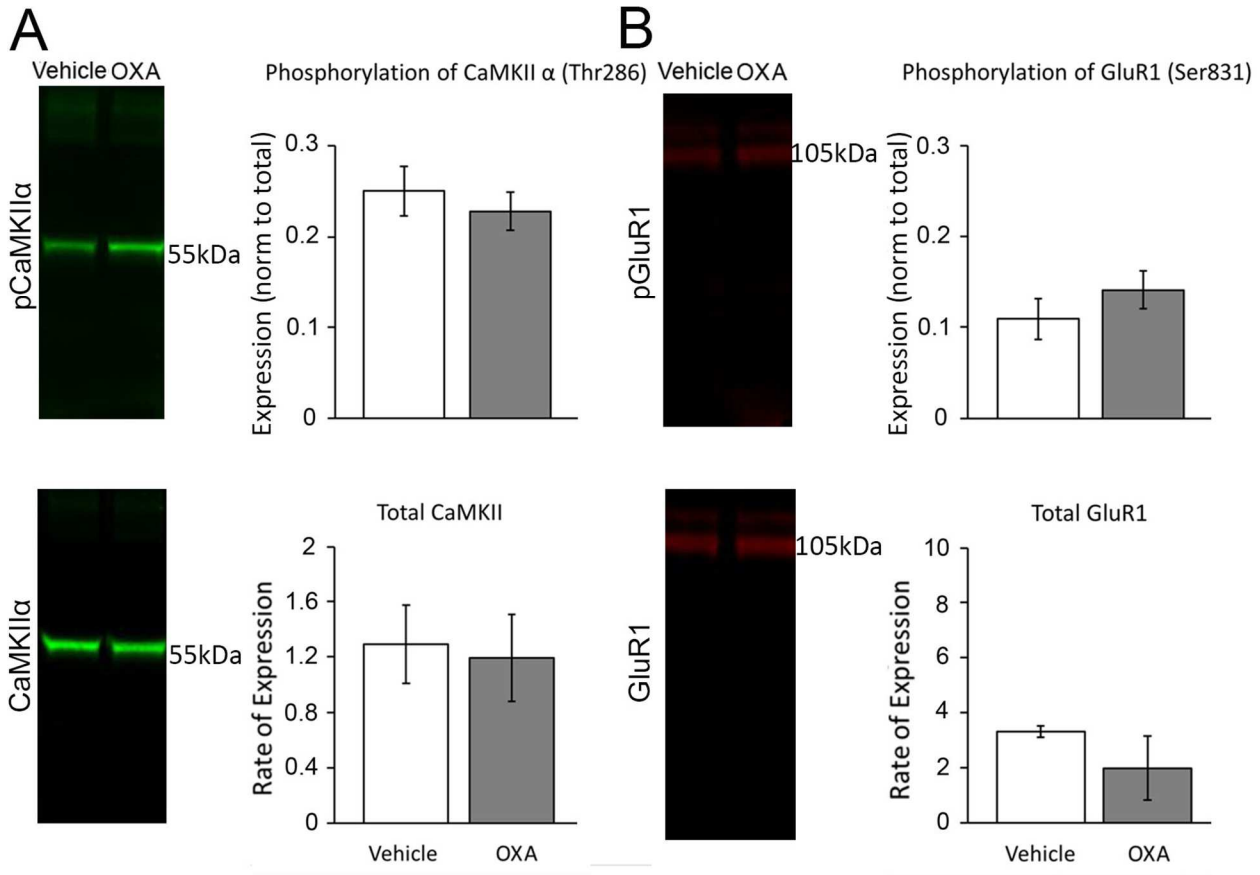

Figure S3.3 CaMKII and GluR1 phosphorylation remain unchanged after a single administration of intranasal OXA following training of MWM. (A) There were no significant differences in either CaMKIIα(Thr286) ratio of phosphorylation or total CaMKIIα expression upon administration of OXA during the retrieval phase of MWM testing (n=8/condition; independent-samples *t*-test; CaMKIIα(Thr286)- $t(14)= -0.645$, $p>0.05$; CaMKIIα- $t(14)= -0.549$, $p>0.05$). (B) No significant differences were detected in either GluR1(Ser831) ratio of phosphorylation or total GluR1 expression (GluR1(Ser831)- $t(14)= 1.052$, $p>0.05$; GluR1- $t(14)= 0.976$, $p>0.05$). Data are represented as mean ± SEM.

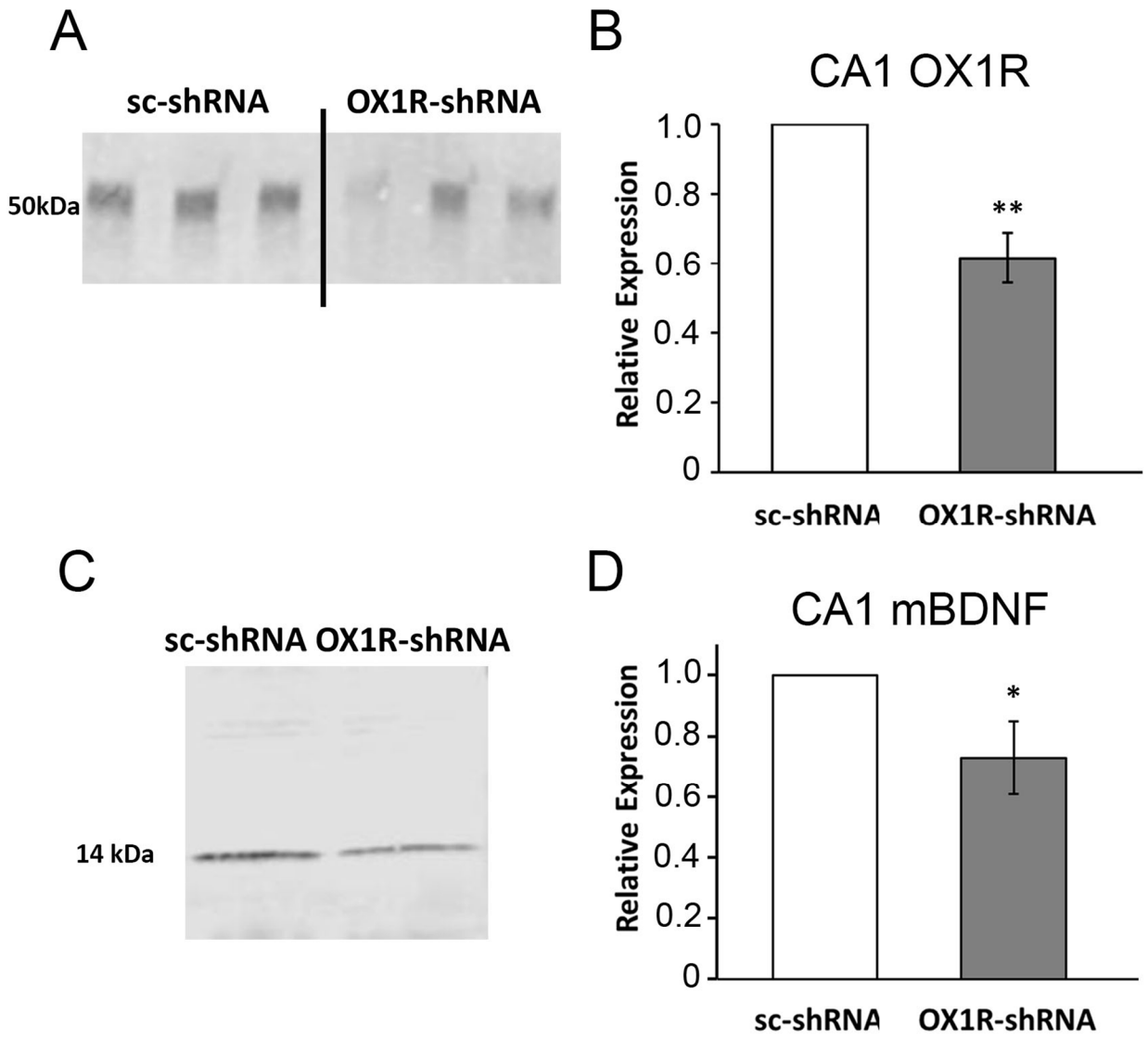

Figure S3.4 Knockdown of OX1Rs in the dHPC and mBDNF expression. (A) Photomicrograph of an OX1R Western Blot for animals introduced with the knockdown or scrambled control vectors. (B) dHPC sites that were injected with the rAAV1-OX1R-shRNA vector showed lower levels of OX1R expression compared to sites injected with the control vector (n=6/condition; independent-samples *t*-test; $t(10)= 5.338$, $p<0.001$). (C) Representative photomicrograph of a Western Blot displaying bands for mBDNF expression in the dHPC for knockdown and control conditions. (D) dHPC sites that were injected with rAAV1-OX1R-shRNA exhibited significantly lower levels of mBDNF expression compared to sites injected with the control vector (n= 6/condition; Mann-Whitney U test; $U= -2.051$, $p<0.05$). Data are represented as mean ± SEM. *$p<0.05$, **$p<0.001$.

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