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TIMING OF LIGHT PULSES AND PHOTOPERIOD ON THE DIURNAL RHYTHM OF HIPPOCAMPAL NEURONAL MORPHOLOGY OF SIBERIAN HAMSTERS

T. IKENO, [a,b]* Z. M. WEIL [b] AND R. J. NELSON [b]

[a] Department of Psychology, Michigan State University, East Lansing, MI 48824, USA

[b] Department of Neuroscience, The Ohio State University Wexner Medical Center, Columbus, OH 43210, USA

Abstract—Rapid remodeling of neurons provides the brain with flexibility to adjust to environmental fluctuations. In Siberian hamsters, hippocampal dendritic morphology fluctuates across the day. To reveal the regulatory mechanism of diurnal remodeling of hippocampal neurons, we investigated the effects of light signals applied under different photoperiodic conditions on dendritic morphology. A 4-h dark pulse during the morning of long days (LD) increased basilar dendritic length, as well as complexity of basilar dendrites of neurons in the CA1. A light pulse during the late night in short days (SD) reduced basilar dendrite branching and increased primary apical dendrites of CA1 neurons. Spine density of dentate gyrus (DG) dendrites was increased by a dark pulse in LD and spine density of CA1 basilar dendrites was decreased by a light pulse in SD. These results indicate that light signals induce rapid remodeling of dendritic morphology in a hippocampal subregion-specific manner. A light pulse in SD decreased hippocampal expression of *fetal liver kinase 1* (*Flk1*), a receptor for vascular endothelial growth factor (VEGF), raising the possibility that VEGF–FLK1 signaling might be involved in the rapid decrease of branching or spine density of CA1 basilar dendrites by light. **© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: diurnal rhythm, hippocampus, neuroplasticity, neurotrophic factor.

INTRODUCTION

Although long-term stability of the neuronal system is important for proper brain function, rapid remodeling of neurons is also necessary for the brain to cope with the changing environment. One of the most plastic sites in adult mammalian brains is the hippocampus, which plays crucial roles in learning, memory formation, and cognitive behaviors (Breedlove and Jordan, 2001). In response to various external and internal conditions, such as stressors (Watanabe et al., 1992; Chen et al., 2008; Magariños et al., 2011), hibernation (Popov et al., 1992; Magariños et al., 2006), reproductive status (Gould et al., 1990; Woolley et al., 1990), and photoperiod (Pyter et al., 2005; Workman et al., 2011; Walton et al., 2013), hippocampal neurons undergo structural alterations in dendrite generation, growth, branching, and spine formation.

Hippocampal dendritic patterning and spine density undergo diurnal fluctuations that are influenced by photoperiod in Siberian hamsters (Ikeno et al., 2013). A diurnal rhythm in neuronal morphology within the infralimbic cortex, an area implicated in cognitive function, has also been reported (Perez-Cruz et al., 2009). These findings suggest that rapid regulation of neuronal architecture contributes to synchronizing neuronal function to external daily cycles. However, the regulation of diurnal rhythms of dendritic alterations is not fully understood.

Because daily environmental fluctuations are derived from light–dark conditions, most diurnal rhythms are acutely affected by photic signals through phase shifting of the circadian clock, an endogenous mechanism generating internal circadian rhythms, or through the direct masking of clock outputs (Ko and Takahashi, 2006). Clock-regulated synthesis and release of the pineal hormone melatonin are inhibited by light, and melatonin acts as temporal cues for the circadian clocks located in various tissues (Pevet and Challet, 2011). Melatonin also plays an important role in the photoperiodic response, because the duration of melatonin secretion represents the length of night, which varies seasonally (Pevet and Challet, 2011). In seasonal mammals including Siberian hamsters, which undergo regression of reproductive organs in short days, suppression of nighttime melatonin secretion by light in short days triggers reproductive activation by inducing the long-day pattern of the hypothalamic genes important for the gonadotropin regulation (Barrett and Bolborea, 2012). Recent evidence has suggested that melatonin has a neuroprotective function and stimulates dendritogenesis and spinogenesis (González-Burgos et al., 2007; Ramirez-Rodriguez et al., 2011; Domínguez-Alonso et al., 2012) via its

*Corresponding author. Address: Department of Psychology, Michigan State University, 217 Giltner Hall, East Lansing, MI 48824, USA. Tel: +1-517-432-5414; fax: +1-517-432-2744.
E-mail addresses: ikenotom@msu.edu (T. Ikeno), zachary.weil@osumc.edu (Z. M. Weil), randy.nelson@osumc.edu (R. J. Nelson).
Abbreviations: BDNF, brain-derived neurotrophic factor; DG, dentate gyrus; Flk1, fetal liver kinase 1; GnRH, gonadotropin-releasing hormone; LD, long-day conditions; SD, short-day conditions; TrkB, tropomyosin receptor kinase B; VEGF, vascular endothelial growth factor; ZT, zeitgeber time.

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actions as free radical scavenger and antioxidant (Reiter, 1998) and via its modulatory action on cytoskeleton organization (Benítez-King, 2006). Melatonin also increases neurotrophic factors in cultured neurons and in the hippocampus (Imbesi et al., 2008; Soumier et al., 2009). Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF), have been implicated in neuroplasticity by signaling through their receptors, tropomyosin receptor kinase B (TrkB) and fetal liver kinase 1 (FLK1), respectively (McAllister, 2001; Carmeliet and Ruiz de Almodovar, 2013). Taken together, these studies raise the possibility that remodeling of hippocampal dendrites depends on external photic signals, which are mediated by nocturnal secretion of melatonin.

In the present study, we investigated the acute effects of a dark pulse during the early morning of long days and a light pulse during the late night of short days on dendritic morphology of hippocampal neurons to reveal the mechanism underlying the diurnal rhythm of neuronal morphology in male Siberian hamsters. We also investigated mRNA expression of neurotrophic factors, *Bdnf* and *Vegf*, and their receptors, *trkB* and *Flk1*, in the hippocampus to investigate the possibility that the dendritic remodeling was mediated by neurotrophic factor signaling.

EXPERIMENTAL PROCEDURES

Animals

Siberian hamsters (*Phodopus sungorus*) used in this study were bred in our colony at The Ohio State University. Male hamsters were weaned during the light phase at 21–24 d of age and immediately placed into either short-day conditions (SD: 8-h light–16-h dark) or maintained in long-day conditions (LD: 16-h light–8-h dark; lights off at 15:00 h EST in all cases) and at a constant temperature of 21 ± 2 °C and relative humidity of 50 ± 10%. Hamsters were individually housed in polypropylene cages (30 × 15 × 14 cm) and had *ad libitum* access to food (Harlan Teklad Rodent Diet 8640; Indianapolis, IN, USA) and filtered tap water. All procedures were approved by the Ohio State University Institutional Animal Care and Use Committee and comply with guidelines established by the National Instituted of Health published in *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources (U.S.), 2011).

Experimental design

The experimental design is depicted in Fig. 1. Hamsters were housed in their respective photoperiods for 5 weeks (N = 10 in LD; N = 11 in SD). During the light phase of the experimental day, five animals of the LD group were transferred to SD to expose them to dark at the early light phase (a dark pulse). At ZT (zeitgeber time) 4 (4 h after light on) in LD and corresponding time in LD with a dark pulse, animals were killed and tissues were collected. Six animals of the SD group were transferred to LD to expose them to light at the late dark phase (a light pulse). At ZT20 in SD and SD with a light pulse, animals were killed and tissues were collected.

Tissue collection and processing

Hamsters were anesthetized with isoflurane vapors and rapidly decapitated. Testes were removed and weighed. Brains were removed and cut along the anterior–posterior axis by using a razor blade. Right and left hemispheres were chosen randomly and half of the brain was placed in RNA later (Ambion, TX, USA) and held at 4 °C to maintain mRNA integrity for gene expression analysis, and the other half was processed for Golgi impregnation using the FD Rapid GolgiStain Kit (FD NeuroTechnologies Inc., MD, USA) according to the manufacturer's instructions.

Analysis of neuronal morphology

Brains were sliced at 100 μm on a cryostat and counterstained with cresyl violet (Sigma–Aldrich, MO, USA). Hippocampal cell morphology was assessed in the CA1 and dentate gyrus (DG) fields in the dorsal hippocampus. Sections were visualized using a Nikon E800 brightfield microscope and intact neurons were reconstructed using Neurolucida software (MicroBrightField, VT, USA) with a 20× objective. Six representative neurons were traced per area, from each animal. The criteria for neuronal selection were: (1) neurons had to be fully impregnated, (2) dendrites could

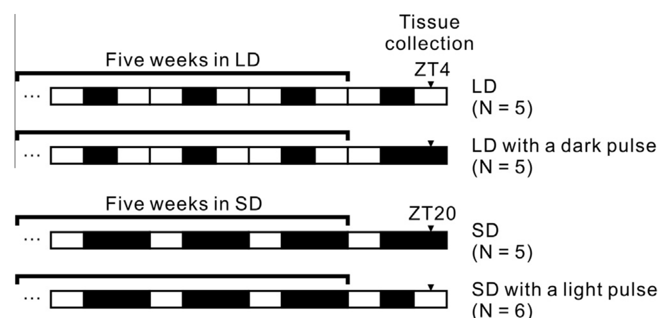

Fig. 1. Experimental design. The white and black bars show the light and dark phases, respectively. Hamsters housed in long-day conditions (LD) for 5 weeks were transferred to short-day conditions (SD) to expose them to dark pulse during the early light phase (a dark pulse), or those housed in SD for 5 weeks were transferred to LD to expose them to a light pulse during the late dark phase (a light pulse). At zeitgeber time 4 (ZT4) in LD or ZT20 in SD and corresponding times in LD with a dark pulse and SD with a light pulse, animals were killed and tissues were collected.

not be truncated, and (3) for the CA1 region, neurons had to be pyramidal cells with somata lying within the pyramidal cell layer and for the DG, neurons had to be granule cells with somas lying within the granule cell layer. The cell traces were analyzed using the accompanying NeuroExplorer software (MicroBrightField). The number of branch points, total length of dendrites, and mean length of dendritic segments (intervals between soma origin and the first branch point, between two successive branch points, or between the last branch point and the branch end) were calculated as previously described (Ikeno et al., 2013). Sholl analyses were also conducted. In our analyses, the distance between concentric Sholl circles was set to 10 μm. The number of intersections of dendrites and Sholl circles was counted as previously described (Ikeno et al., 2013).

For spine density analysis, six neurons (in both the pyramidal cell layer of the CA1 and granule cell layer of the DG) were selected from each animal. Spines were counted on dendrites longer than 20 μm if they were beyond at least one branch point using Neurolucida software with a 100× objective. Spine density (spines/ 1 μm) was calculated for each trace in NeuroExplorer software.

Analysis of gene expression

The expression levels of *Vegf*, *Flk1*, *Bdnf*, and *trkb* in the hippocampus were investigated. The hippocampus was dissected from one brain hemisphere by gently separating it from the overlying cortex and the underlying thalamus, and then total RNA was isolated using Trizol (Life Technologies, CA, USA). DNA in RNA samples was digested with deoxyribonuclease I, Amplification Grade (Life Technologies). cDNAs were synthesized from 0.5 μg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). For real-time PCR analysis, 1% of the cDNA was used at a final concentration of 1× Power SYBR Green PCR Master Mix (Life Technologies) and 0.05 μM of each primer using a 7500 Fast Real-Time PCR System (Life Technologies). Each reaction was performed in duplicates. Primer sequences for *Vegf*, *Bdnf*, *trkb*, and *18S rRNA* were reported previously (Ikeno et al., 2013). Sequences of forward and reverse primers for *Flk1* were 5′-TGG TT CCC TGG TCG TGA AT-3′ and 5′-CAG GAG AGA TCA AGG CTT TCT CA-3′, respectively. In all the reactions, the generation of only a single expected amplicon was confirmed by melting analysis. Quantification of cDNAs was performed by the standard curve methodology.

Statistical analysis

Reproductive measures, neuronal measures, and gene expressions were analyzed by *t*-tests. A repeated measures ANOVA was used for the data from Sholl analysis. Statistics were performed using GraphPad Prism 4 (GraphPad Software, Inc., CA, USA). Mean differences were considered statistically significant when $P < 0.05$.

RESULTS

Reproductive measures

Hamsters exposed to SD for 5 weeks after weaning had significantly smaller paired testes mass than hamsters exposed to LD ($t_{(8)} = 16.0$, $P < 0.0001$). There were no significant differences between LD and LD with dark pulse conditions ($t_{(8)} = 0.694$, $P > 0.05$) and between SD and SD with light pulse conditions ($t_{(9)} = 1.177$, $P > 0.05$).

Dendritic morphology

The number of branch points in basilar dendrites of CA1 pyramidal neurons was significantly reduced by a light pulse in the late dark phase (ZT20) of SD ($t_{(64)} = 2.663$, $P < 0.05$; Fig. 2A). However, there were no significant effects of light conditions on branch point number in apical dendrites of CA1 neurons ($t_{(58)} = 0.927$, $P > 0.05$ for LD, and $t_{(64)} = 0.500$, $P > 0.05$ for SD; Fig. 2A) or dendrites of DG granule neurons ($t_{(58)} = 0.973$, $P > 0.05$ for LD, $t_{(64)} = 0.669$, $P > 0.05$ for SD; Fig. 2A). Total length of dendrites of CA1 basilar dendrites was significantly increased by a dark pulse in the early light phase (ZT4) of LD ($t_{(58)} = 2.101$, $P < 0.05$; Fig. 2B). Those of CA1 apical dendrites and DG dendrites were not significantly changed by a dark pulse in LD ($t_{(58)} = 1.293$, $P > 0.05$, and $t_{(58)} = 0.280$, $P > 0.05$, respectively) or a light pulse in SD ($t_{(64)} = 0.389$, $P > 0.05$, and $t_{(64)} = 0.137$, $P > 0.05$, respectively; Fig. 2B). The number of primary apical dendrites of CA1 neurons was significantly increased by a light pulse in SD ($t_{(64)} = 2.249$, $P < 0.05$; Fig. 2C). A dark pulse in LD significantly increased the total number of intersections between CA1 basilar dendrites and Sholl circles ($t_{(58)} = 2.175$, $P < 0.05$; Fig. 3A). A repeated measures ANOVA also showed a significant increase in intersections induced by a dark pulse ($F_{(1,36)} = 4.731$, $P < 0.05$; Fig. 3A). There were no significant effects of a light pulse in SD on the number of intersections in CA1 basilar dendrites ($t_{(64)} = 1.417$, $P > 0.05$ Fig. 3A). The number of intersections of CA1 apical dendrites was not significantly affected by light conditions ($t_{(58)} = 1.159$, $P > 0.05$ for LD, $t_{(64)} = 0.289$, $P > 0.05$ for SD; Fig. 3B). There was no significant effect of dark or light pulses on the number of intersections of DG dendrites ($t_{(58)} = 0.285$, $P > 0.05$ for LD, $t_{(64)} = 1.826$, $P > 0.05$; Fig. 3C).

Spine density

The spine density of CA1 basilar dendrites was significantly reduced by a light pulse exposure in SD ($t_{(262)} = 3.367$, $P < 0.01$; Fig. 4). On the other hand, a dark pulse in LD significantly increased the spine density of DG dendrites ($t_{(238)} = 3.436$, $P < 0.01$; Fig. 4).

Gene expression

The dark pulse in LD did not significantly affect mRNA expression levels of neurotrophic factors or their receptors *Vegf* ($t_{(8)} = 0.702$, $P > 0.05$), *Flk1*

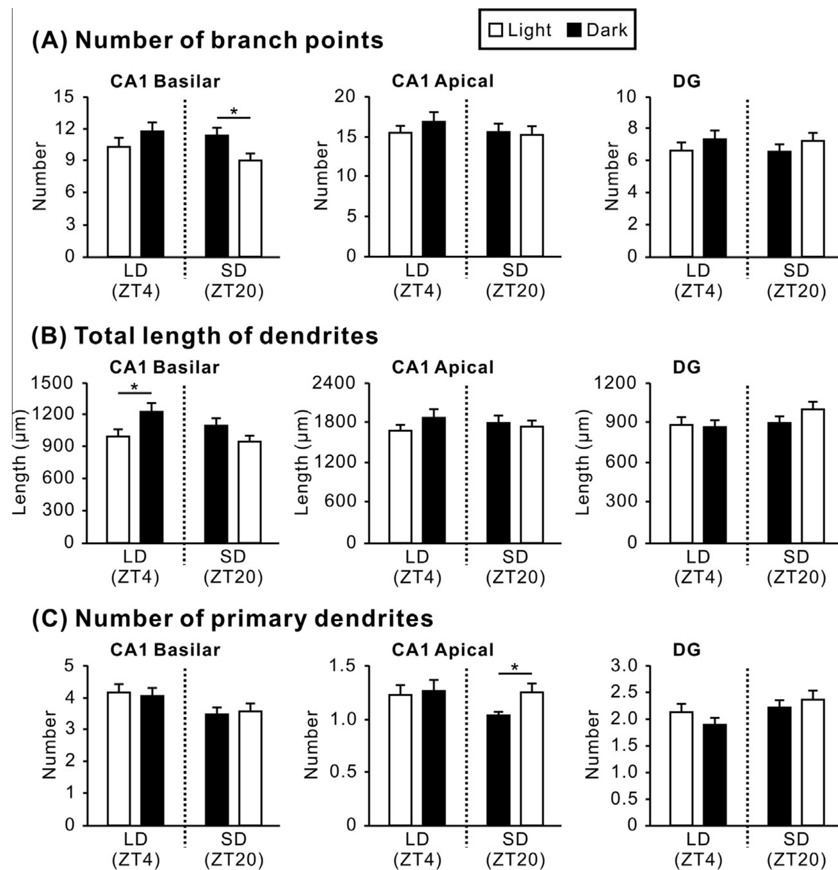

Fig. 2. Effects of a dark pulse in long-day conditions (LD) and a light pulse in short-day conditions (SD) on (A) the number of branch points, (B) dendritic length, and (C) the number of primary dendrites of CA1 neurons (basilar and apical dendrites) and of dentate gyrus (DG) neurons. $^{*}P < 0.05$ (t-test).

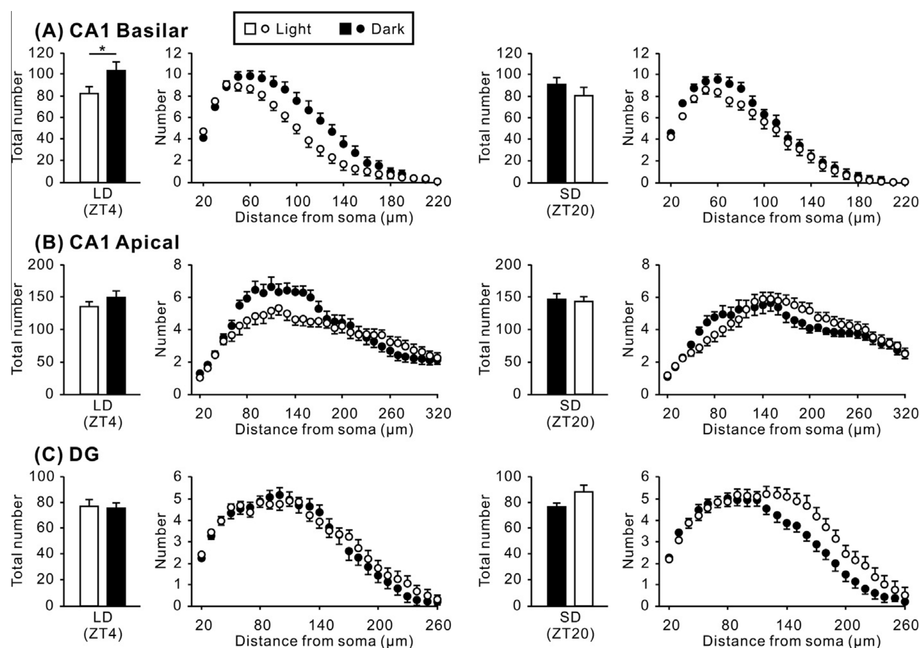

Fig. 3. Effects of a dark pulse in long-day conditions (LD) and a light pulse in short-day conditions (SD) on the total number of intersections with Sholl circles (left) and the number of intersections with each Sholl circle (right) of (A) basilar dendrite of CA1 neurons, (B) apical dendrites of CA1 neurons, and (C) dendrites of dentate gyrus (DG) neurons. $^{*}P < 0.05$ (t-test).

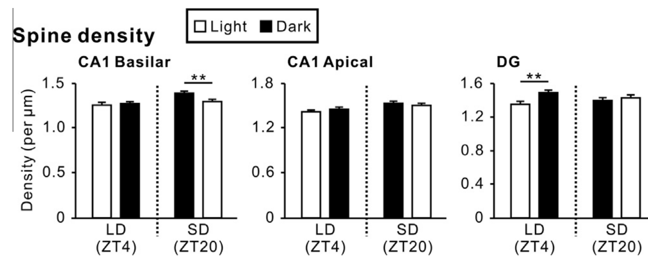

Fig. 4. Effects of a dark pulse in long-day conditions (LD) and a light pulse in short-day conditions (SD) on spine density of basilar and apical dendrite of CA1 neurons and dendrite of dentate gyrus (DG) neurons. $^{**}P < 0.01$ (*t*-test).

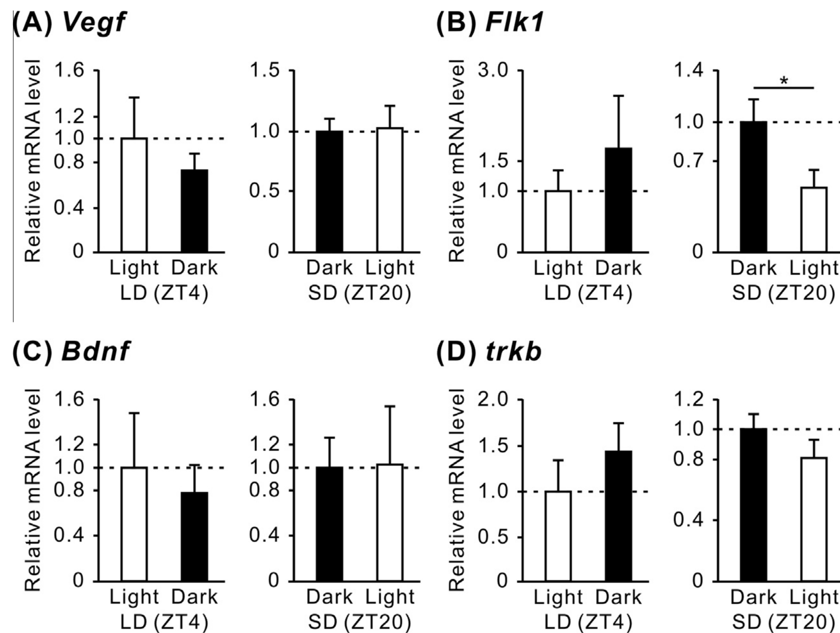

Fig. 5. Effects of a dark pulse in long-day conditions (LD) and a light pulse in short-day conditions (SD) on mRNA expression of (A) *Vegf*, (B) *Flk1*, (C) *Bdnf*, and (D) *trkb* in the hippocampus. Mean values in LD or SD were set at 1.0. $^{*}P < 0.05$ (*t*-test).

($t_{(8)} = 0.758$, $P > 0.05$), *Bdnf* ($t_{(8)} = 0.401$, $P > 0.05$), and *trkb* ($t_{(7)} = 0.928$, $P > 0.05$) in the hippocampus (Fig. 5). The light pulse in SD also did not affect expression of *Vegf* ($t_{(8)} = 0.912$, $P > 0.05$), *Bdnf* ($t_{(8)} = 0.080$, $P > 0.05$), and *trkb* ($t_{(8)} = 1.142$, $P > 0.05$) (Fig. 5A, C, D); however, *Flk1* expression was significantly reduced by a light pulse ($t_{(8)} = 2.339$, $P < 0.05$; Fig. 5B).

DISCUSSION

The present study demonstrated that short-term changes in light conditions induced structural alterations in hippocampal neurons. A dark pulse in the early morning, or extension of night, in LD increased the complexity of the CA1 neurons' structure with longer dendrites and more intersections with Sholl circles. On the other hand, a light pulse during the late night in SD reduced dendrite branching of CA1 neurons. We previously showed that hippocampal neuronal morphology of Siberian hamsters undergoes diurnal changes; in CA1 neurons, dendrite length and intersections with Sholl circles increase during the dark phase in LD, whereas dendrite branching decreases during the light phase in SD (Ikeno et al., 2013). Thus, our current data suggest that light and dark pulses affected neuronal morphology through the phase shift of the circadian clock regulating the diurnal morphological changes or through the direct effect on dendritic patterning.

Spines constitute the postsynaptic site for most excitatory inputs (Bourne and Harris, 2008). Therefore, higher spine density leads to the larger number of synaptic inputs to the neurons. Given that the increase in dendritic characteristics, such as total branches and lengths, also contributes to the increase in synaptic input on the dendrites, it is reasonable to suggest that structural alterations in dendritic patterning are coincident with alterations in spine densities as demonstrated by studies using various experimental conditions (Magariños et al., 2006, 2011; Lin et al., 2012; Parihar and Limoli, 2013). However, although a decrease in spine density of CA1 basilar dendrites by a light pulse in SD was consistent with a decrease in branch points of the dendrites, the stimulatory effect of a dark pulse in LD on spine density of DG neurons was not observed on any characteristics

of dendritic patterning of these neurons. These results suggest that dendritic patterning and spine density are regulated by a distinct mechanism and light signals affect these mechanisms differently. Previously, we reported that spine density of CA1 dendrites, especially apical dendrites, increased during the light phase in LD but during the dark phase in SD, but that of DG dendrites increased during the light phase in SD (Ikeno et al., 2013). However, here we demonstrated that light conditions did not affect spine density of CA1 apical dendrites, and moreover, spine density of DG dendrites was increased by a dark pulse in LD. These present results cannot be simply explained by the effects of light conditions on the diurnal rhythm of spine density, suggesting that light and dark pulses have direct effects on spine density in a subregion-specific manner and not through the mechanism driving the diurnal rhythmicity in the spine density.

Neurotrophic factors have been implicated in neuroplasticity (McAllister, 2001; Carmeliet and Ruiz de Almodovar, 2013). Here we discovered that not a dark pulse in LD, but a light pulse in SD significantly reduced the mRNA expression of *Flk1*, which encodes the receptor of VEGF, but *Vegf* expression was not affected. These results raise the possibility that decrease of the FLK1 proteins as a result of decreased mRNA by a light pulse limits VEGF actions, leading to neuronal morphological changes, especially reduction of dendritic branching or spine density in CA1 neurons, which were reduced only by a light pulse in SD. Indeed, importance of *Flk1* expression for VEGF signaling in regulation of spinogenesis in hippocampal neurons was previously reported (Huang et al., 2012). No significant effects of a light pulse on *Bdnf* and *trkb* mRNA expression were observed, suggesting that transcriptional regulation of these genes are not involved in light signal-induced rapid remodeling of hippocampal neurons. However, it remains possible that the morphological changes demonstrated here require post-translational modifications of BDNF, such as the cleavage of proBDNF to mature BDNF, which plays a key role for the action of BDNF (Waterhouse and Xu, 2009).

One of the possible factors controlling dendritic morphological changes is melatonin. Recent evidence has indicated that melatonin contributes to neuroplasticity by promoting dendrite and spine formations (González-Burgos et al., 2007; Ramirez-Rodriguez et al., 2011; Domínguez-Alonso et al., 2012). Moreover, the positive effect of melatonin on neurotrophic factor levels has been also demonstrated (Imbesi et al., 2008; Soumier et al., 2009). These studies support the hypothesis that rapid remodeling of dendritic structure observed in the present study was caused by the suppression and induction of nocturnal melatonin by light and dark pulses, respectively. In seasonal mammals, duration of melatonin secretion is used as photoperiodic information, and therefore, a light manipulation to shorten the melatonin-secreting period in SD is interpreted as a LD signal, leading to activation of the reproductive axis (Barrett and Bolborea, 2012). It was previously shown that *gonadotropin-releasing hormone* (*GnRH*) mRNA expression in the hypothalamus was induced within few hours after exposing Siberian hamsters to LD (Porkka-Heiskanen et al., 1997). Although SD conditions used in the present study inhibited puberty, it is still possible that a light pulse applied during the late night of SD induced a LD-specific hormonal state. Sex steroid hormones (estradiol and its metabolic product testosterone) have been proposed to modulate hippocampal synapse formation (Woolley et al., 1990; Leranth et al., 2000, 2003; Kovacs et al., 2003). In addition to gonadal synthesis, GnRH may also directly stimulate estradiol synthesis in the hippocampus and the local estradiol synthesis may change hippocampal spine synapse density (Prange-Kiel et al., 2013). Hippocampal subregional differences in the expression of steroidogenic acute regulatory protein (StAR) and aromatase, enzymes important for estradiol synthesis (Prange-Kiel et al., 2006), also support the subregion-specific patterns in dendritic morphological changes (in the present study; Ikeno et al., 2013). Therefore, the discrepancy between acute changes in spine density induced by light and dark pulses and diurnal changes shown previously might be derived from different syntheses of estradiol, or testosterone, in response to the photoperiodic regulation on GnRH.

CONCLUSION

Our results indicate that a dark pulse during early morning of LD and a light pulse during late night of SD induced rapid changes in dendritic patterning and spine density of hippocampal neurons. Decreased *Flk1* expression in the hippocampus might support the view that the morphological changes induced by a light pulse resulted from reduced VEGF–FLK1 signaling.

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