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Original Article

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Transcranial Light Alters Melanopsin and Monoamine Production in Mouse (*Mus musculus*) Brain

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Abstract

Background: The mammalian circadian system sets a rhythm for the appropriate occurrence of physiological and behavioral phenomena during a 24-h period. Since the duration of the circadian system is usually less or more than 24 h, it must be entrained regularly and light is the governing stimulus of the rhythm. The target for light stimulus is the master circadian clock, which is located in the suprachiasmatic nucleus in the hypothalamus. One of the key molecules transmitting light information and entraining the clock is melanopsin (OPN4), a G protein-coupled molecule that is found most abundantly in the retina and brain. Although light stimulus is usually mediated through the eyes, light has an ability to penetrate the skull. Here, we present the effect of transcranial light illumination on OPN4 and serotonin expression in the mouse brain.

Methods: Male mice were randomly assigned to a control group, morning-light group and evening-light group, and animals were illuminated transcranially five times a week for 8 min for a total of 4 weeks. The concentrations of OPN4 and monoamines were analyzed with Western blot and high-performance liquid chromatography (HPLC) techniques, respectively.

Results: Our results show that transcranial light illumination increases the amount of OPN4 in the hypothalamus and cerebellum. Additionally, the production of serotonin in the cortex was shown to decrease in the morning-light group.

Conclusions: With this study, we provide novel information on the effects of light administration through the skull on transmitters regulating circadian rhythmicity by showing that transcranial light affects molecules involved in circadian rhythmicity.

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Introduction

Light is the main signal that entrains the mammalian circadian system. This system is evolved to a set rhythm, in which physiological and behavioral events occur during a 24-h period. Since the circadian clock is a free-running system, and produces a rhythm even when environmental cues are absent [1], it is not exactly 24 h, and thus must be entrained regularly to ensure accordingly timed physiological phenomena [2]. If the light stimulus is diminished or absent, the circadian clock is disturbed and several psychoneurological disorders, such as seasonal affective disorder (SAD) in humans, may occur [3]. Furthermore, mice have also been shown to have SAD-like behavior, when light conditions are irregular [4].

The mammalian master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Its main purpose is to generate and synchronize the circadian rhythms of peripheral tissues. According to a generally accepted assumption, the only route by which light signals reach the hypothalamus is through the eyes. This hypothesis suggests that intrinsically photosensitive retinal ganglion cells (ipRGCs) gather light information in the retina and project it to the SCN through the retinohypothalamic tract (RHT) [2, 5, 6]. One of the key molecules for retinal phototransduction is melanopsin (OPN4) [6-9], although it is not crucial for these events since multiple types of photoreceptors are capable of transmitting light stimuli [7].

Phototransduction is not necessarily restricted to the RHT. It has been shown that mammalian brain consists of photosensitive opsin proteins also outside the RHT [10-13], and that light penetrates the mammalian skull [14-17]. It has been also proved that light is capable of phosphorylating OPN4, which inhibits the G protein-coupled activation [18], although only some OPN4 gene variants have been shown to affect the responses to light stimulus [19]. Notwithstanding general criticism, we have recently shown that it is possible to stimulate brain opsins by illuminating the brain via ear canals [20].

OPN4 is a part of a large and old family of heptahelical light-sensitive G protein-coupled transmembrane receptors, which consist of a protein constituent and a chromophore. Opsins are expressed mostly in the brain and in the retina of

mammals [21, 22]. The opsin-mediated phototransduction mechanism can be divided into two steps: absorption of light by the retinal part of the opsin protein and the subsequent light-initiated photoisomerization from 11-cis to all-trans conformation [23]. This conformational change allows activation of the G protein and the phototransduction cascade [2, 24].

OPN4 was found for the first time in the light-sensitive melanophores in frog skin as well as in the brain SCN and the eye [25]. Furthermore, OPN4 has been found in all animal groups, including mammals, and it functions as a photoisomerase [26, 27]. The functions of OPN4 are very different from the other photosensory opsins [24], since it is also required for non-visual photic responses [28] in the eye. In mammals, OPN4 is the most abundant photopigment of ipRGCs and it is a key molecule in mediating the effects of light on the brain via RHT [29]. OPN4 is an important factor for maintaining circadian rhythms also, although not crucial for entrainment in all mammalian species [7, 30, 31].

Besides opsins, monoamines are also shown to affect circadian rhythmicity. Many external factors influence the monoamine concentration in the hypothalamus, including season, the time of the day, and the intensity of light [32, 33]. Apart from the brain, light also changes the monoamine concentration in the peripheral tissues. The extraneuronal monoamine transporter has been known to be an important factor in a signal transmission chain as an agent transferring monoamine-based information to peripheral tissues [34].

As mentioned above, if the photoperiod of an animal is shortened, certain physiological conditions may occur. Besides the mood alterations, SAD has been shown to increase body weight due to increased carbohydrate foraging and decreased physical activity [35, 36]. Bright light treatment is used as a therapy method for helping SAD symptoms. It decreases body weight, especially due to reduction of body fat, in obese humans regardless of the seasonality trait [37]. Furthermore, it has been shown that rhythm-related gene loci affect long-term changes in energy expenditure [38], but the direct effects of transcranial light on the changes energy expenditure have not yet been studied.

As shown, OPN4 is expressed in the vertebrate brain [25, 27, 39], and opsins can be stimulated transcranially [20]. Additionally, monoamines play a role in light signaling [40], and also e.g. in SAD symptoms [41]. In order to determine whether transcranial light has direct effects on OPN4 and monoamine expression in the brain at different times of the day, we illuminated mice transcranially either in the morning or in the evening. With the results of this study, we are able to show that light through the skull plays a role in activating molecules that are associated with circadian rhythmicity.

Materials and Methods

Animals

Thirty (30) adult C3H/HeNHsd rd/rd male mice (*Mus musculus*, Harlan Laboratories, Venray, The Netherlands) were used in this study, which was approved by the Finnish National Committee for animal experimentation (license number ESAVI/567/04.10.03/2012). The animals were 8 - 10 weeks old at the beginning of the study. The mice used in the study were visually blind. Their rods had degenerated 5 weeks postnatal and cones secondarily, which leads to adult blindness [42], but the ganglion cell layer is functional.

Experimental procedure

All the mice were kept in a 12:12 LD rhythm. The light period of the mice started at 7:00 and finished at 19:00. Mice were randomly assigned to three different groups: control (CONT), morning-light group (ML) and evening-light group (EL). The animals were weighed three times a week. Transcranial light was given via ear canals for 4 weeks, five times a week. Light treatment was given under anesthesia (isofluran, anesthesia 3%, maintenance 1.5%) for 8 min per mouse. The ML group was exposed to light just after the beginning of the light period (treatment time 7:30 - 9:30) simulating the time when mice are typically inactive, and the EL group was exposed just after the beginning of the dark period (treatment time 19:00 - 21:00), simulating the time when mice are typically active [43]. The intensity of the light in the headphones of the light source (Valkee NPT 1000, Valkee Inc., Oulu, Finland) was 2.00×10^{15} photons/cm^2/s. We have presented the spectrum of the headphones in our previous article with an intensity peak at approximately 450 nm [20]. There is no significant heat effect caused by the device, since only a non-measurable small fraction of the mechanical heat produced by the light source (totally less than 14.7 mW) is conducted into the ear canal. The CONT group was anesthetized and headphones were inserted, but no light was given. After 4 weeks of light treatment, mice were killed by cervical dislocation, the length and body mass of the animals were measured and samples of cortex, hypothalamus, cerebellum, liver, retina and plasma were collected between 8:00 and 14:00 and stored at -80 °C. Body mass indexes (BMI) were calculated using the general BMI formula: BMI = mass/length^2. Masses were described as kilograms and length as meters.

SDS-PAGE and Western blotting

Total protein concentrations of the brain tissue samples were defined according to Bradford [44]. Homogenization buffer (62.5 mM Tris-HCl, pH 6.8), containing leupeptin (1 µg/mL), pepstatin A (1 µg/mL) and PMSF (1 mM) was added to the samples and homogenized (Qiagen TissueLyser, Retsch, Haan, Germany).

Samples with equal amounts of protein (12.06 µg/lane) were loaded in separating gel (4-12%, Amersham™ ECL™ Gel, GE Healthcare Bio-Sciences, Uppsala, Sweden) and electrophoretically separated at 160 V for 60 min. The separated proteins were transferred to a nitrocellulose membrane (0.45 µm, Bio-Rad Laboratories, USA) according to the method of Towbin et al [45].

After electroblotting, the membranes were blocked with 5% non-fat milk powder in TBS for 1 h at room temperature. After washing (3 × 5 min) in TBST (TBS + 0.05% Tween-20), the membranes were incubated in primary antibody for mel-

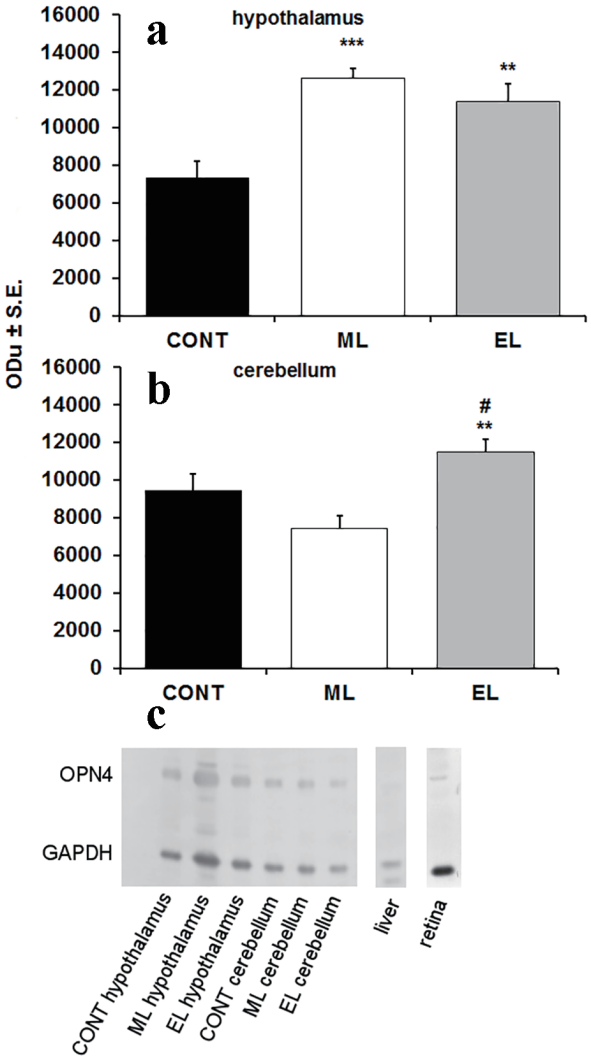

Figure 1. Expression of OPN4 in hypothalamus and cerebellum of mouse brain. (a) A bar graph summarizing results from Western blot analysis showing mean optical density (ODu) of OPN4 in relation to GAPDH in hypothalamus (N = 10 samples per group). **P < 0.01, ***P < 0.001; error bars indicate SE. (b) A bar graph summarizing results from Western blot analysis showing mean optical density (ODu) of OPN4 in relation to GAPDH in cerebellum (N = 10 samples per group). **P < 0.01; error bars indicate SE. # marks the significance compared to ML group, ##P < 0.001. (c) A representative Western blot membrane showing expression of OPN4 with a molecular weight of 53 kDa. GAPDH was used as a loading control with a molecular weight of 37 kDa. CONT: control group; ML: morning-light group; EL: evening-light group.

anopsin (molecular weight 53 kDa, anti-melanopsin antibody, ab65641, Abcam, Cambridge, UK) for 2 h at room temperature. Melanopsin antibody was diluted in TBST (1:500). Then the membranes were washed (3 × 5 min) and incubated with a secondary antibody (1:3,000, Bio-Rad Goat Anti-Rabbit IgG (H+L)-AP-Conjugate, Bio-Rad Laboratories, Hercules, USA) in TBST for 2 h at room temperature. Antibody detection was performed with bromo-4-chloro-3-indolyl phosphate mono-(-toluidinium) salt/ nitro blue tetrazolium (BCIP/NBT) substrate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, molecular weight 37 kDa) was used as a loading control (GAPDH (14C10) Rabbit mAb, Cell Signaling Technology, Danvers, USA). To measure gel loading, membranes were stripped using stripping buffer (1.5 g glycine, 0.1 g SDS, 1 mL Tween-20 diluted in 100 mL ultrapure water; pH 2.2). Membranes were incubated 10 min with stripping buffer, followed by 2 × 10 min wash with PBS and 2 × 5 min wash with TBST. After washing, the stripped membranes were blotted as before. GAPDH was diluted in TBST-antibody solution (1:1,000) and incubated for 2 h with the primary antibody. Immunoreactive band intensities were analyzed using the VersaDoc imaging system (Bio-RadUSA). Liver was used as a negative control.

Monoamine concentration analysis by high-performance liquid chromatography (HPLC)

Monoamine concentrations were determined by HPLC in plasma and adrenal gland samples, as described by Nieminen et al [46]. The 5-HT samples were analyzed using the same technique. Briefly, brain samples were deproteinized before injection into the HPLC autosampler. The running buffer contained 50 mM NaH_2PO_4, MetOH and aluminum chlorohydrate in 77:15:8 ratios, respectively (pH 3.22). The running buffer was filtered and 200 mg Na-dodecylsulphate and 77.4 mg Na-EDTA were added to the buffer. The total concentration of base solution was 5 µg/mL in 0.1 M $HClO_4$.

Statistical analysis

In this study, all values are expressed as mean ± standard error (SE). For the amounts of OPN4, monoamines and melatonin, one-way ANOVA was used for comparisons between groups. In case of significant changes, pairwise comparisons between both the test group and CONT group were made using the Tukey posterior test. The length, weight and BMI comparisons between groups were also made with one-way ANOVA and Tukey posterior test. Weight changes within groups were compared using a paired *t*-test.

Results

Melanopsin protein abundance in the mouse hypothalamus and cerebellum

Western blot was used to analyze the abundance of OPN4 in mouse hypothalamus and cerebellum. The used antibody detected a single band of approximately 53 kDa, similar to the predicted protein size of OPN4. In hypothalamus samples, significant differences were observed between control and experimental groups in the OPN4 content. Compared to the CONT group, the amount of OPN4 was 1.72-fold higher in the ML group (P < 0.001), and 1.54-fold higher in the EL group (P < 0.01) (Fig. 1a). The amount of OPN4 in the cerebellum was 1.21-fold higher in the EL group (P < 0.01), but no differences

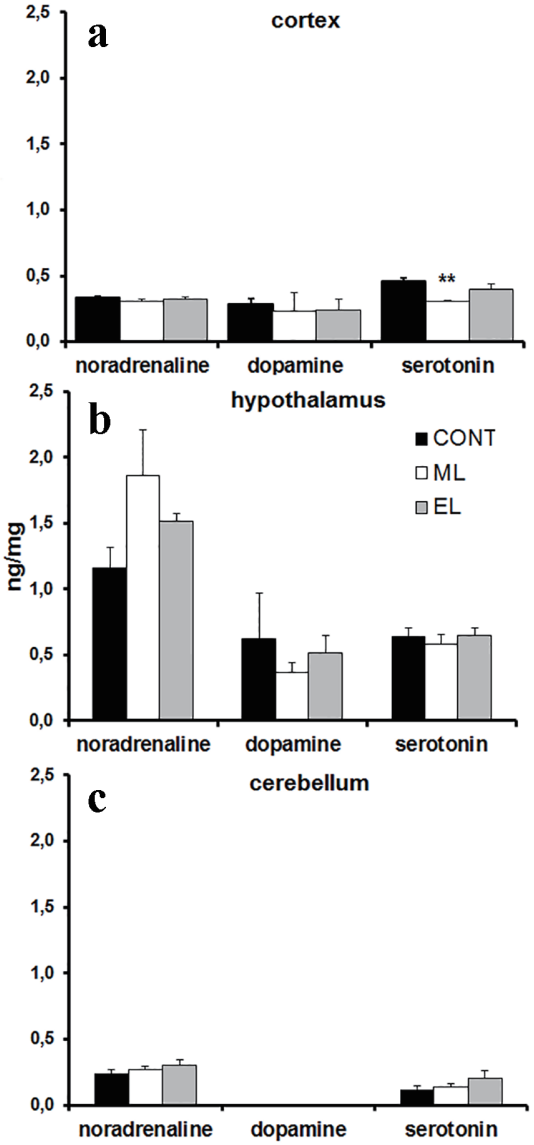

Figure 2. Monoamine concentrations in mouse cortex, hypothalamus and cerebellum. (a) A bar graph summarizing results from HPLC analysis showing mean ng/mg in cortex. N = 10 samples per group. **P < 0.01. (b) A bar graph summarizing results from HPLC analysis showing mean ng/mg in hypothalamus. N = 8 - 10 samples per group. (c) A bar graph summarizing results from HPLC analysis showing mean ng/mg in cerebellum. N = 8 - 10 samples per group. CONT: control group; ML: morning-light group; EL: evening-light group. Error bars indicate SE.

were seen in the ML group compared to controls. Additionally, compared to the ML group, the amount of OPN4 was 1.55-fold higher in the EL group (P < 0.001) (Fig. 1b). A Western blot showing the OPN4 expression in different groups and tissues is represented in Figure 1c.

Monoamines in the mouse brain

The amounts of adrenaline, noradrenaline, dopamine and serotonin in mouse cortex, hypothalamus and cerebellum were analyzed using the HPLC method. Melatonin concentrations were analyzed from plasma samples. The amount of serotonin in the cortex was 0.66-fold lower (P < 0.01) in ML mice compared to the controls (Fig. 2a), but there were no other differences between the experimental group monoamines and the control group monoamines (Fig. 2b, c). There was no detectable amount of adrenaline in the brain samples. There was no measurable amount of melatonin in any groups. No changes were seen in BMIs between groups at the end of the study.

Discussion

In this study, we describe the direct effects of transcranial light on OPN4 and monoamine expression in the mammalian brain. We were also able to show the connection between transcranial light and circadian rhythmicity, since the results of the expression level of OPN4 in the cerebellum differed between the test groups. There were significant differences both in OPN4 and 5-HT expression levels related to the time when the light was administered. This study showed that the amount of OPN4 increased significantly in transcranially illuminated mice when compared to controls. Furthermore, the 5-HT production in the cortex decreased when mice were transcranially illuminated in the morning.

Based on the findings of this study, light-activated molecules in the brain can be stimulated with transcranial light. In the hypothalamus, the amount of OPN4 in transcranially illuminated mice was higher than in the controls, and the amount of OPN4 was higher regardless of the duration of illumination. The commonly acknowledged and best-known route for light-mediated effects is through the eye, where OPN4-containing ipRGCs gather light information and deliver it to the SCN via RHT [6, 47, 48]. Besides the retina, OPN4 is also found in the vertebrate brain [25, 39] along with other opsins [10, 12, 13, 39]. Additionally, OPN4 is considered to be an important molecule in adjusting the master clock and entraining the circadian rhythm [7-9, 28], and light has been shown to act as a direct modulator for OPN4 actions [30]. Therefore, it is reasonable to hypothesize that transcranial light entrains the circadian system by enhancing OPN4 production in the hypothalamus. Since OPN4 is known to have an important role in light transmission through the eyes, and on the other hand, OPN4 is shown to be expressed in the hypothalamus [27], it may be capable of activating circadian signaling pathways directly by transcranially penetrated light, although we acknowledge that there might be some light transmitted through the transcranially illuminated ocular photoreceptors [49, 50]. Even though the absorption maximum of our light source (450 nm) [20] was not optimal for the OPN4 expression maximum (476 - 484 nm) [51, 52], we were able to obtain significant results in this study.

The time of the illumination had an effect on the amount of OPN4 in the cerebellum also. We found that the expression of OPN4 was significantly higher in the EL group, but no differences were found in the ML group compared to controls. Moreover, the amount of OPN4 was significantly higher in the EL group when compared to the ML group. OPN4 has been

found in the vertebrate cerebellum [27, 39]. Whether light has a direct effect on its expression in the cerebellum has not yet been clarified. It has also been shown that the cerebellum is involved in maintaining the circadian rhythm in rodents through clock gene regulation [53, 54], and OPN4 resets the circadian clock through the activation of clock gene Per1 [55]. Therefore, we must consider the possibility that OPN4 may affect the circadian rhythm through clock gene activation in the cerebellum. The amount of OPN4 mRNA in the brain is highest in the late subjective night in avian species [39]. Our results suggest a similar occurrence in protein level in mouse cerebellum, as we know that mice are nocturnal animals, and the amount of OPN4 was highest in the EL group. Furthermore, recent research by Kumar et al hypothesizes that myelinated fibers may be capable to transmit light information in the brain [56]. If true, transcranial light may have also direct effect on cerebellum through these fibers.

Transcranial illumination decreased the amount of serotonin in the cortex of ML group mice compared to controls. The function of 5-HT is regulated by monoamine oxidase A (MAO-A), which is an important factor in metabolizing monoamines [57]. In general, if the amount of MAO-A increases, the amount of serotonin decreases [58]. There was a similar pattern in cortical noradrenaline and dopamine in the ML group also, although the changes were not statistically significant. Additionally, the changes in monoamine concentrations may refer to circadian rhythm disturbations [59]. It should be remembered that mice are nocturnal animals and the ML group was illuminated in the beginning of their resting state, which might disturb the circadian rhythm and stress the animals. We have previously published results regarding the effects of transcranial light on monoamine concentrations in plasma and adrenal gland samples [20]. We showed that the levels of dopmanine and noradrenaline increased, and the levels of adrenaline decreased with transcranially illuminated mouse [20]. These results suggest that the experiment created long-term stress effects, which may be caused by the actual experiment or the disturbations in the circadian rhythm. Moreover, the serotonin concentration is shown to peak at the end of the light phase in rodent brain [60-62]. Therefore, we must consider the possibility that the sample collection time was not optimal for serotonin concentration levels in this study.

Owing to our study design, we cannot be sure about the causal relationship between OPN4 and 5-HT concentration. However, based on the results of this study, it is reasonable to hypothesize that the change in cortical 5-HT levels may be linked to the increased OPN4 expression in the brain. OPN4 transfers visual information to the thalamo-cortical pathway, which is involved in many events in the brain [63]. If OPN4 regulates this pathway, it may also affect cortical functions by decreasing 5-HT production in the cortex of ML group mice. However, the level of knowledge regarding the relationship between 5-HT and OPN4 is still low, and the relationship between these two compounds should be further studied. In this connection, it has also to be reminded that another monoamine, i.e., DA has earlier been shown to regulate OPN4 expression in ganglion cells [40]. Also, given that OPN4 gene variants have earlier been shown to be associated in SAD [19], the putative OPN4 mediated treatment effect of transcranial bright light on SAD remains also to be investigated.

In conclusion, this is the first study to show that transcranial light has a significant effect on OPN4 expression in the mouse brain. We were also able to alter the serotonin levels in the cortex with transcranial illumination. This research indicates that molecules influenced by circadian rhythmicity in the brain can be stimulated by light through the skull via ear canals. We only studied the changes in OPN4 expression at the protein level. Therefore the changes at the OPN4 mRNA level would be of interest in future studies, although it is already known that the levels of mRNA and protein have similar pattern in normal lighting conditions in the rodent retina [64]. Additionally, with the used methods, we cannot say if the changes in OPN4 levels are caused by expression level changes inside the cells or by the total amount of OPN4 expressing cells. It would also be important to study closely, whether the changes in OPN4 levels are caused by changes in monoamine levels and vice versa.

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Competing Interests

Antti Flyktman has received a small grant from Valkee Inc., Juuso Nissila is a minor shareholder of Valkee Inc. Toni Jernfors, Satu Manttari, Markku Timonen and Seppo Saarela have no competing interests in relation to this study.

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