MOLECULAR MECHANISMS OF CIRCADIAN REGULATION DURING SPACEFLIGHT

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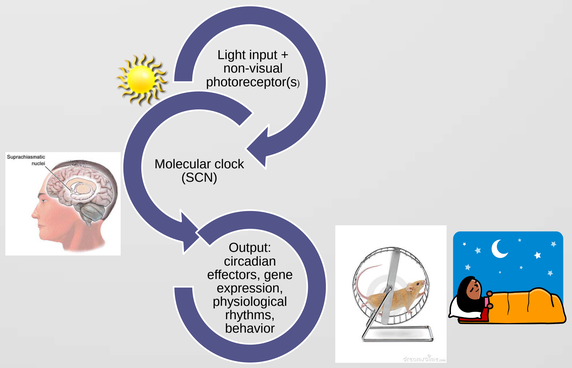
INTRODUCTION

The physiology of both vertebrates and invertebrates follows internal rhythms coordinated in phase with the 24-hour daily light cycle. This circadian clock is governed by a central pacemaker, the suprachiasmatic nucleus (SCN) in the brain. However, peripheral circadian clocks or oscillators have been identified in most tissues. How the central and peripheral oscillators are synchronized is still being elucidated.

Light is the main environmental cue that entrains the circadian clock. Under the absence of a light stimulus, the clock continues its oscillation in a free-running condition. In general, three functional compartments of the circadian clock are defined:



The vertebrate retina contains endogenous clocks that control many aspects of retinal physiology, including retinal sensitivity to light, neurohormone synthesis[1] (melatonin and dopamine), rod disk shedding, signalling pathways and gene expression.

Neurons with putative local circadian rhythm generation are found among all the major neuron populations in the mammalian retina. In the mouse, clock genes and function are more localized to the inner retinal and ganglion cell layers[2]. The photorreceptor, however, secrete melatonin which may still serve a an important circadian signal.

The reception and transmission of the non-visual photic stimulus resides in a small subpopulation (1-3%) or retinal ganglion cells (RGC) that express the pigment melanopsin (Opn4) and are called intrisically photoreceptive RGC (ipRGC). Melanopsin peak absorption is at 420 nm and all the axons of the ipRGC reach the SCN[3].

A common countermeasure for circadian re-entrainment utilizes blue-green light to entrain the circadian clock[4,5] and mitigate the risk of fatigue and health and performance decrement due to circadian rhythm disruption. However, an effective countermeasure targeting the photoreceptor system requires that the basic circadian molecular machinery remains intact during spaceflight.

We hypothesize that spaceflight may affect ipRGC and melanopsin expression, which may be a contributing cause of circadian disruption during spaceflight.

OBJECTIVE

To compare the ipRGC population and melanopsin expression in retinas from mice flown aboard STS-133 with ground controls.

MATERIALS AND METHODS

The STS-133 flight animal experiment consisted of two albino BALB mice per group (and their ground controls, n=3) whose retinas were collected at R+1, R+5 and R+7. Ground controls consisted in vivarium and animal enclosure module (AEM) animals. All mice were maintained in a 12/12 hour light/darkness cycle; standard illumination in the vivarium is approximately 10-fold the illumination in the AEM.

After enucleation, one eye was fixed for paraffin embedding and immunohistochemistry; the contralateral eye was stored in RNA later until processed for RNA isolation.

Immunhistochemistry and immunofluorescence

The oxidative damage marker **8-hydroxydeoxyguanosine (8-OHdG)**, was detected with a polyclonal antibody to 8OHdG (ab10802, Abcam), and a secondary antibody conjugated with peroxidase and AEC (3-Amino-9-ethylcarbazole) as a substrate to develop color. Light microscopy images were analyzed using the open access software Image J, processing all images identically.

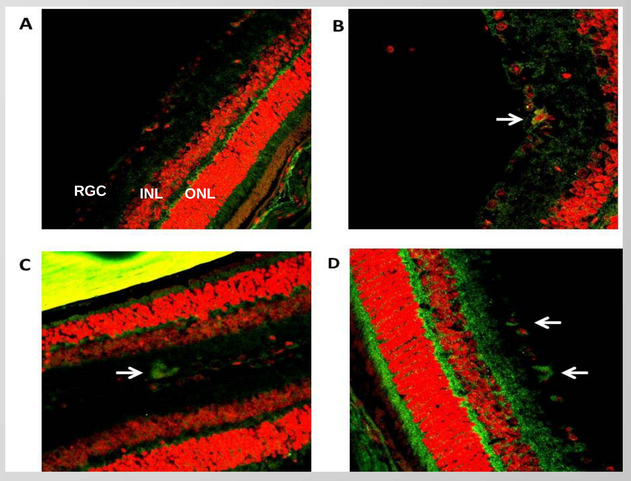
Melanopsin was detected with a rabbit polyclonal antibody that reacts with mouse and rat melanopsin (PA-1-780, Thermo Scientific). Secondary antibody was a goat anti-rabbit IgG conjugated with Alexa 488 (Invitrogen). Images were obtained with a Leica confocal microscope.

Real Time qPCR

Total RNA and DNA were isolated from whole retina using the AllPrep DNA/RNA Micro isolation kit (QIAGEN). cDNA was synthesized using the Quantitect Whole Transcriptome kit (Qiagen) and PCR amplifications were performed using specific primers (Quantitect primers, Qiagen) and SYBR Green as a fluorescent probe (IQ SYBR Green Supermix, BioRad).

RESULTS

Our immunofluorescence results are in agreement with the description of the distribution of ipRGC. RGC positive for melanopsin were found uniformly distributed in the RGC layer throughout the retina, with occasional crowding along the periphery. Virtually no immunoreactive cells were found in retina samples from mice aboard STS133 after one day upon return; however several positive cells were seen in samples from mice after flight on R+7. Likewise, both vivarium and AEM ground controls showed evidence of ipRGC.

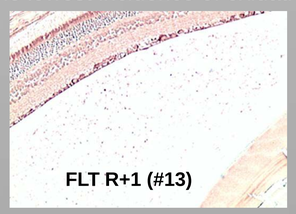


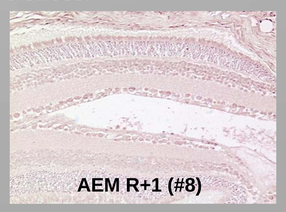
Melanopsin immunofluorescence *(green) in retina sections from BALB mice in the STS133 experiment. White arrows denote positive RGC expressing melanopsin. A: FLT+1 (mouse #13); B: FLT+7 (mouse # 52); C: AEM +1 (mouse #8, note that folded retina is due to an artifact of the preparation); D: VIV+1 (mouse #2). Nuclei are counterstain with the red dye 7-AAD. RGC: retinal ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.*

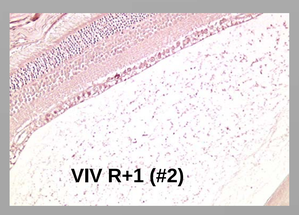
Analysis of melanopsin mRNA levels by real time PCR revealed a drop in melanopsin gene expression in flight samples upon return to Earth, compared to ground controls, but control levels are recovered after 7 days after landing.

Melanopsin (Opn4) gene expression *levels in retina samples from BALB mice in the STS13 experiment, measured by real time qPCR. Y axis: logarithmic scale of comparative gene expression level normalized to housekeeping genes. The numbers in green indicate the mouse # in the experiment. For #13, the expression levels were closest to zero, as pointed by the arrow (qPCR amplification curved shifted to the right, below threshold, so the value that appears in the graph is actually an artificial value close to zero that was necessary to be added in the calculations process).*

In order to investigate the levels of oxidative stress in the samples, the DNA damage oxidative stress marker 8OHdG was qualitatively measured in each immunostained retinal sections. The figure below shows representative fields of retinal sections comparing the DNA damage due to oxidative stress. RGC appear to be a target of damage in flight samples, whereas photoreceptors are more affected in vivarium samples. AEM ground controls showed the lowest incidence of oxidative stress.

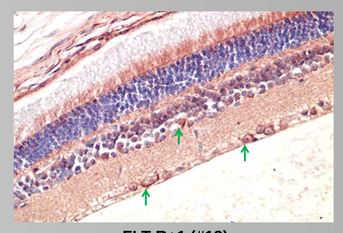


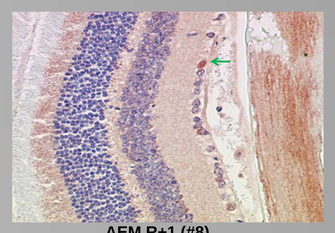


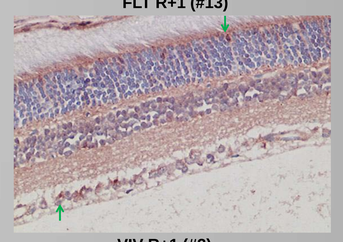


Light microscopy image of 8OHdG immunoreactivity (purple-brown, green arrows) in retina sections of mice aboard STS133 and ground controls, on R+1.

In addition, we investigated whether RGC loss by apoptosis, measured by activated caspase-3 immunoreactivity could be associated with the decrease in melanopsin expression. Conclusive quantification of apoptosis-positive cells is in progress, however, some differences were seen in the apparent distribution of apoptotic RGC in the different samples, being more prevalent in the ONL in vivarium samples and in the INL and RGC in flight samples Therefore we infer that cell death may be one of the causes in the decrease of melanopsin expression, besides a downregulation in melanopsin expression itself. In vivarium samples, where cell death was observed in the photoreceptor layer, there is no decrease in melanopsin expression,





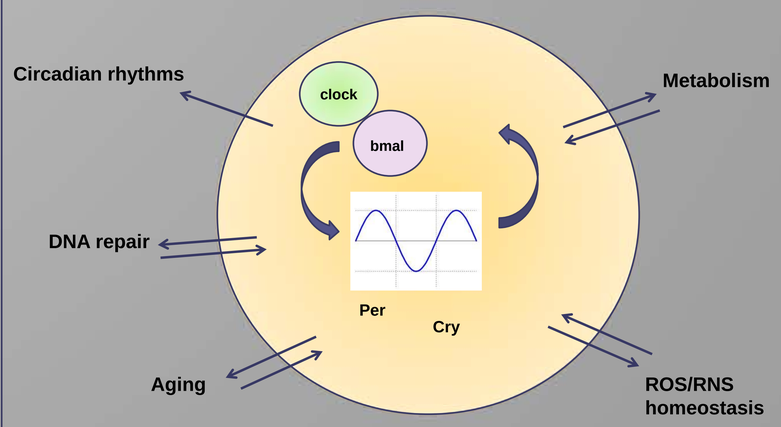


Light microscopy image of caspase-3 immunoreactivity (red-brown, green arrows) in retina sections of mice aboard STS133 and ground controls, on R+1.

CONCLUSIONS

In conclusion, the number of melanopsin-immunoreactive RGC as wel l as melanopsin gene expression were decreased in flight samples immediately after flight but this change was attenuated in flight sample 7 days after return. Retinal ganglion cells are a target of the effects of oxidative stress induced by spaceflight, based on immunohistochemistry of 8OHdG in eye samples. We propose that oxidative stress can lead to a decrease in melanopsin expression, likely via ipRGC loss or impairment, and thus, it can be a contributing factor to circadian disruption during spaceflight. Countermeasures contemplating the use of light should therefore be complemented with melanopsin expression maintenance and/or reduction in oxidative stress.

There is previous published evidence suggesting that the central clock is susceptible to oxidative stress[6], often associated with aging, and that DNA repairing mechanisms and circadian clocks share regulatory pathways. Future questions to be answer include: a) is the decrease in melanopsin expression observed after spaceflight due to RGC loss or to RGC impaired gene expression?; b) are other clock genes also affected?; c) is the local retinal clock output affected?; d) does the decrease in melanopsin translate into a significant alterations in the signaling to the SCN to contribute to circadian rhythm disruption?; e) which retina-specific cellular rhythms might be affected by a local circadian clock disruption?



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ACKNOWLEDGEMENTS

This work was funded by NASA HRP(MTL707). We thank the assistance of Audrey Nguyen, JSC SLSD intern, Clear Lake High School, in the preparation of immunostained slides and image analysis, and James Fiedler, PhD, for help with graphing the gene expression data.