Investigating the molecular mechanisms of melatonin as a humoral zeitgeber in the filamentous fungus *Neurospora crassa*.

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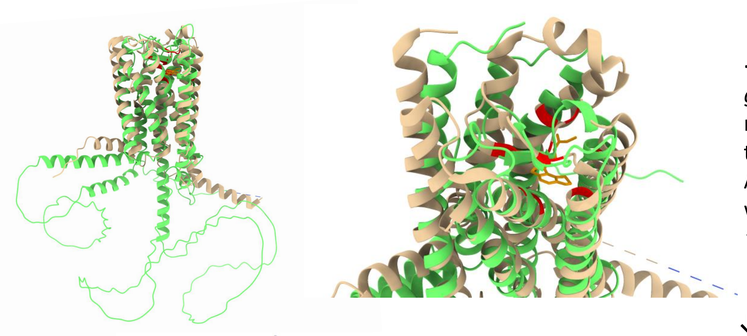
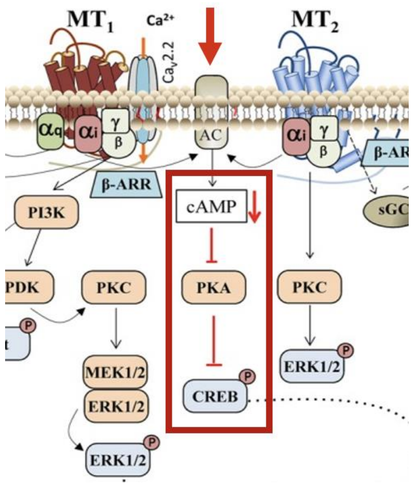
Background and Hypotheses

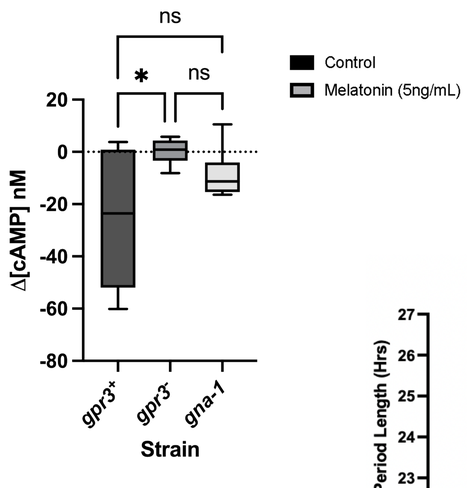
Melatonin is one of the most well documented elements of circadian rhythms in humans and is ubiquitous to all domains of life[1,2]. While in general the end point physiological effects of melatonin are known, much remains to be understood about the molecular mechanisms by which it exerts its effects on circadian regulation. Additionally, little research has been done on the conservation of the melatonin signaling pathway in Eukaryotes. In our research, we aim to provide evidence of conserved melatonin signaling pathways between lower and higher Eukaryotes by revealing the mechanisms of this pathway in the filamentous fungus *Neurospora crassa*. Since finding a putative melatonin-related receptor in *N. crassa* [4], our research is focusing in on the following hypotheses:

1. **The novel fungal melatonin receptor has conserved downstream mechanisms to that of MT1/MT2: 1) changes to negative elements of the molecular circadian oscillator (TTFL), 2) reduction of cAMP**
2. **Given the multiple downstream effectors of melatonin like redox conditions and cAMP signaling, there are GPCR-dependent and independent affects of melatonin on circadian rhythms.**



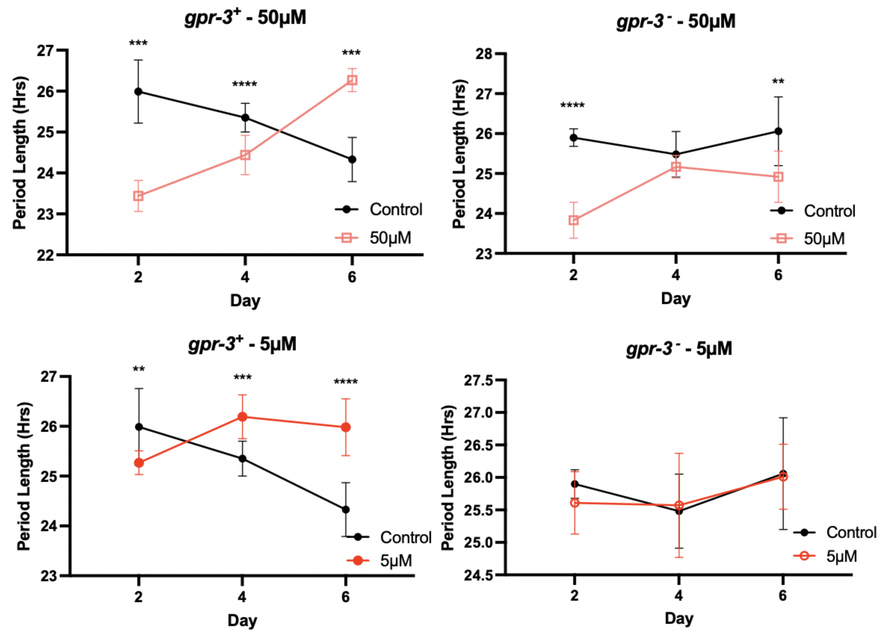
↑ Figure 1. GPR-3 is structurally similar to hMT1 and has predicted melatonin binding Top) Experimental MT1 (tan) and *N. crassa* GPR3 (green). RMSD: 1.213. Residues interacting with melatonin highlighted in red, MT1 agonist Ramelteon bound to experimental MT1 colored orange. **Bottom)** Top-down view of SwissDock binding predication for *gpr-3* and melatonin. Top 5 clusters shown for clarity, AC score: -23.36, SwissParam: -6.88



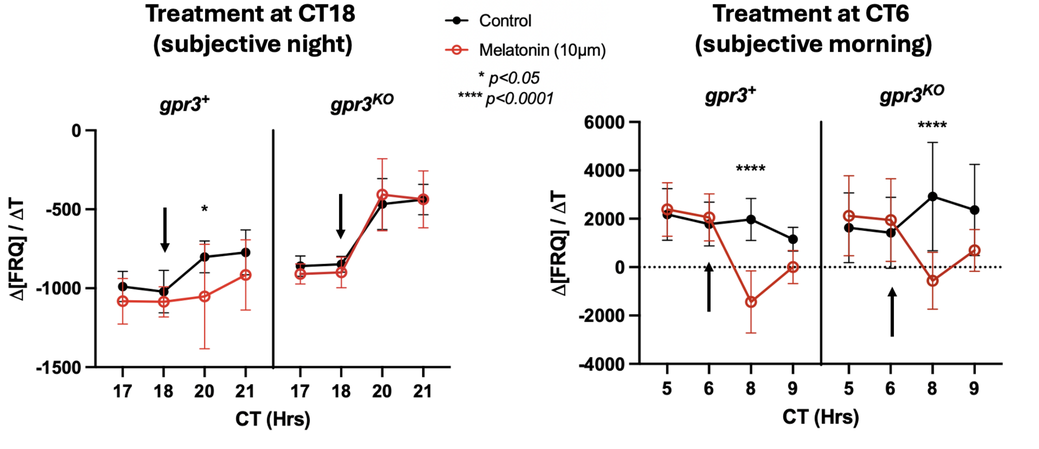


↓Figure 2. *gpr3*- strains are insensitive to melatonin-induced cAMP reduction. ***Left)*** figure taken from Cecon et al. 2018 showing the known cAMP signaling response downstream of hMT1/hMT2 ***Right)*** difference in relative [cAMP] in gpr3 knockout and WT after 10-minute treatment with 5ng/mL melatonin. One-way ANOVA reveals significant decrease in cAMP in WT not experienced by knockouts. *ns - not significant, * p<0.05,* n=6

→Figure 3. Melatonin alters period length in a gpr-3 dependent manner. Wild type and *gpr-3* knockout samples were treated with a high, receptor-independent concentration of melatonin (50μM) or a low, receptor-specific concentration (5μM) at ZT8. Control was treated with plain media. Period length averages were calculated every 48hrs. One-way ANOVA reveals significant biphasic responses to melatonin to both concentrations in the wild type, but only significant non-biphasic responses to high doses in the knockout. ***p<0.01, ***p<0.001, ****p<0.0001.* n=12



↓Figure 4. Melatonin induced phase shifts in FRQ in a time- and receptor-dependent manner. Samples of a wild type and gpr-3 knockout had their periods monitored for when CT18 and CT6 would occur. Samples were removed and treated with 20μL of sterile water (control, black lines) or sterile water with 10μM melatonin (red lines) at **left**) CT18 or **right**) CT6. Average RoC of relative FRQ levels were analyzed using 2-way ANOVA. Black arrows show time of treatment. n=4



Preliminary Conclusions

1. **Knockouts of *gpr-3* are insensitive to melatonin induced: cAMP reduction, period length changes, and FRQ phase shifts**
2. **There is receptor in/dependent affect of melatonin on FRQ phase shifts depending on subjective time of treatment and concentration of melatonin treatment.**
3. **There is a biphasic response to melatonin, shortening period upon initial treatment then lengthening period after roughly 3 days. At low concentrations this is receptor-dependent.**

Methodology and Analysis

ChimeraX Analysis and SwissDock: PBD files were visualized and individually aligned in ChimeraX using the "matchmaker" command (Needleman-Wunsch alignment). PBD file for *gpr-3* was uploaded to SwissDock along with the structure of melatonin. The structure was searched at (-8,5,9) with 1 RIC.

FRQ::LUC Assay: Strains were grown as mycelial pads in high glucose media (HGM) in LD12:12 conditions for two days. Samples were punched out of the mycelial pad and used to inoculate a 96 well plate. This was put into constant dark in the luminometer. Luminescence from fusion protein of luciferase and Frequency was recorded. **Figure 3-** at ZT8 (8hrs after lights on) samples were treated with plain LGM media or 50μM or 5μM of melatonin in LGM. Samples were then left in luminometer, and data was collected for 7-8days. 48hr period averages were calculated using BioDare. **Figure 4-** Cells had their rhythms monitored and were treated with either water or 10μM melatonin in water at either CT6 or CT18 and luminescence was recorded hourly. Relative change in Frequency protein was determined using the raw RLU values of two time points and calculating rate of change over time.

cAMP Assay: Strains were grown on complete media slants in LD12:12 for 5 days. At ZT12 a conidia suspension was made for each strain using HGM and left to culture until ZT18. Cells were treated with either sterile water or 5ng/mL melatonin for 10min. Conidia were pelleted, lysed, and purified in preparation for use. At this point, the 96 well format for the Promega cAMP Glo™ assay kit was followed. Relative change in cAMP levels calculated from standards as outlined in the assay protocol. n=6 for each group[4].

Acknowledgements

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[1]Zhao, D. et al. 2019. Melatonin Synthesis and Function: Evolutionary History in Animals and Plants. Frontiers in Endocrinology 10: 249
[2]Balzer, I., Höcker, B., Kapp, H., Bartolomaeus, B. (2000). Occurrence and comparative physiology of melatonin in evolutionary diverse organisms. In: Driessche, T.V., Guisset, JL., Petiau-de Vries, G.M. (eds) The Redox State and Circadian Rhythms. Springer, Dordrecht.
[3]Cecon, E., Oishi, A., & Jockers, R. (2018). Melatonin receptors: molecular pharmacology and signalling in the context of system bias. British journal of pharmacology, 175(16), 3263–3280.
[4]Maienza, C. S. D., Lamoureux, G., & Lee, K. (2025). Cross-species comparison of AlphaFold-derived G protein-coupled receptor structures reveals novel melatonin-related receptor in Neurospora crassa. PloS one, 20(1)