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Shining a light on the parasitic manipulation of Rhodopsin by fungi in *Camponotus floridanus*

Abstract

There are several known parasites that can influence the behaviour of their host, but the mechanisms underlying this manipulation are still unknown. Recently research has revealed the sequences of proteins that are involved in different stages of infection of *Camponotus floridanus* by *Ophiocordyceps camponoti-floridani*. There is little to no information about how these proteins actually work. The behaviour caused by the fungi is well documented: after infection the ant stops interacting with its peers and leaves the nest. It displays problems in walking and eventually climbs a plant and bites down on the stem. Here it stays while the fungi uses its body as sustenance and uses the elevated position to scatter it spores again. In this proposal we request funding for experiments surrounding Rhodopsin of the *Camponotus floridanus*. We theorize that this receptor of the GPCR family plays a role in the manipulated behaviour during infection. We look to prove interaction between the fungal proteins found in previous research by our lab and rhodopsin. We will start this search with informatics, followed by yeast-2-hybrid. Following this we will prove a link between the proteins that have shown an interaction in our previous assays and the manipulated behaviour by injecting the proteins in live ants and finally we will try to confirm which pathways are affected by RNAi in live ants, looking for the same behaviour. With this our hypothesis is that we won't only show the molecular interactions of ant manipulation by *Ophiocordyceps*, but also shine a light on other parasitic manipulation involving light-sensitivity.

Layman Summary

Er zijn verschillende soorten, van bacteriën, schimmels en wormen, die een parasiet relatie hebben met de host die ze infecteren. Sommige van deze parasieten kunnen invloed hebben op het gedrag van hun host, waardoor ze ook wel zombie-parasieten worden genoemd, maar hoe ze dit doen is grotendeels onbekend. Recentelijk zijn eiwitten van host en parasiet gevonden die tijdens de infectie en manipulatie van de parasiet schimmel *Ophiocordyceps* een rol zouden kunnen spelen. *Ophiocordyceps* infecteert mieren en beïnvloedt deze om minder contact te maken met andere mieren en om het nest te verlaten. Hierna klimt de mier een plantenstengel op en bijt zich hier vast terwijl hij langzaam als voedsel wordt gebruikt door de schimmel, waarna de hoogte wordt gebruikt voor de verspreiding van de schimmels sporen. Daarnaast is er aangetoond dat tijdens de infectie van de parasiet de mier slechter op licht reageert, waardoor hun ritme verandert. Ook is de hypothese dat het verlaten van het nest en de hoogte van het klimmen worden bepaald door de hoeveelheid licht. Wij willen in dit onderzoek kijken naar een specifiek eiwit van de gastheer mier genaamd Rhodopsin en zien hoe deze eiwitten geproduceerd door Ophiocordyceps een interactie aan gaan met deze receptor. We verwachten dat deze receptor een rol speelt in de manipulatie, omdat deze word geactiveerd door licht. Om dit te bewijzen willen we een paar experimenten uitvoeren. We beginnen met het modelleren van de interactie met data beschikbaar over de eiwitten, en confirmeren interacties met een gistcel-systeem. Daarna produceren we de gevonden eiwitten en injecteren deze in mieren om te kijken of ze het verwachtte gedrag veroorzaken. Hierna proberen we erachter te komen welke processen in de mier niet meer werken door deze 1 voor 1 uit te schakelen door een techniek genaamd RNA-Interference. Zo hopen we een licht te schijnen op een klein onderdeel van de mechanismen van host-manipulatie.

Keywords

Camponotus floridanus, Ophiocordyceps camponoti-floridani, GPCR, Rhodopsin,

Topic

There are several known species of parasites that are known to influence their hosts in specific behavioural patterns. One of these is *Ophiocordyceps camponoti-floridani*. This fungal parasite is known for its manipulation of infected ants. which together with other parasites causing this extended phenotype has given them the colloquial name of zombie parasites (1) (2).

The behaviour as observed after infection by *Ophiocordyceps* is as follows: ants lose the ability to communicate with their peers and participate less in foraging. (3)(4) The ants also show loss of motor control, leading to a drunkards walk. (3)(5) They will eventually leave the nest and start climbing plants and bite down into the stem, so-called summiting behaviour. The ant stays here, its mandibles clenched down while the fungus uses its body as food to grow until it spreads it spores on the forest floor to infect more ants. (3)(4)(6)(7)

As stated earlier, *Ophiocordyceps* is not the only parasite that influences its host in this kind of way. It is a phenomenon which is spread over several different types of parasites and hosts, like the baculoviruses infecting silkworms. They cause the worm to climb plants and move an exaggerated amount. This causes the worm to get eaten, which continues the viruses' lifecycle in the bird. (8) Another example is the lancet liver fluke. This parasite infects ants as well, and causes them to climb plants, but only during times of low-sunlight.(9) The goal of the fluke is to be eaten by cows, continuing its life-cycle, but the ant dying due to overheating would be detrimental. While these kinds of manipulation have been known for a while,

even in the general population with movies and video games using the concept, little is known about how these parasites actually accomplish this feat. (10)

Only recently more insight into the mechanism of this manipulation by *Ophiocordyceps* has been gained through genomics and machine learning research into the expression of fungal proteins during different stages of infection. (11)(12)(13) One of the protein groups that came forward through this research are proteins that interact with G-protein Coupled Receptors (GPCRs). (11)(13) This is not surprising, because GPCRs are involved in many signalling pathways in eukaryotic cells, receiving signals from neurotransmitters, hormones, pheromones and light. (14)(15) This is a set of proteins, comprised of 7 transmembrane helices with a cytosolic C terminus and an external N-terminus. They are coupled to a G-protein, comprised out of 3 subunits. Effector function of these receptors mostly works through binding of their ligand, which leads to confirmation changes and cleaving of the G-protein in its α, β and γ subunits which have their own downstream cascades within the cell. (14) To make differences between these proteins clearer they are divided in several subgroups, mainly based on the way that activation works in molecular detail. One of those subgroups that we want to look closer at is Class A, also called the rhodopsin-like GPCRs.(15) This group contains Rhodopsin, a GPCR which is located in the eyes and is responsible for turning light signals in the rods of the eyes into reduction of cyclic GMP. This opens channels and increases transmitter release, making us see light, especially in low-light situations. An earlier paper by Will et al (13) used protein prediction software to look at Protein-Protein Interactions (PPI's) between secreted proteins of *Ophiocordyceps* and the entire *Camponotus floridanus* proteome. In this Research they found 16 secreted proteins which showed enrichment when compared to GPCR's in the ant proteome. Large part of these GPCR's were part of the Rhodopsin class of GPCR's. (13)

Another reason why rhodopsin is of particular interest to us is because of the implication that light can play in the manipulated behaviour. (6) Part of earlier research showed that the summiting behaviour is influenced by the hosts daily rhythm. (16) Additionally, the fact that the ant leaves the burrow and loses the sense of its circadian rhythm could suggest a lessened sensitivity to light. (5)(17)(18) Other research showed that the position of summiting is also influenced by light, through amount of cover of the canopy. (19) Furthermore, it is described that spore transmission of the fungi is dependent on certain factors, one of them being light levels. (20) It would therefore be advantageous for the fungi to regulate the amount of light the ant will prefer during the later stages of infection. The role of light is also something we observe in other parasites that induce summiting behaviour, as described in baculoviruses infecting silkworms to move to higher branches or the lancet liver fluke, which induces summiting in ants only during nights.(8)(9) Interesting to note here is the fact that most of these parasites have shown high specificity for host-infection, but these comparable extended phenotypes still developed in several of them.(21) If we can prove that one of the GPCR's found in our omics data (a.k.a Rhodopsin) is being affected by these parasites, the case can be made that other parasites use these as well, due to the observed phenotypes and the high conservation of GPCR's in the genome. (13)(22)(23)

Thus, the reason we expect rhodopsin to play a part in this process is two-fold. First is the fact that we hypothesize that manipulation of rhodopsin would explain the role of light in the extended phenotype as described above. Secondly we saw a significant expression change in the group of GPCR's that rhodopsin is a part off, giving us further cause to believe they play a role. When we review these facts, our interest in this research strengthens. We will explore specific proteins produced by *Ophiocordyceps* which can interact with the Rhodopsin receptor of the host *Camponotus floridanus*. Gaining more insight in how this parasite interacts with the proteins of the host will not only give us information about the molecular

working, but might also give us insight in the evolutionary push within this group of zombie parasites and how they or other parasites using these pathways might be combated. (21)
One of the approaches we want to use for this has recently made significant progress and is the use of machine learning. The amount of PPI's involved in something like *Ophyocordyceps* manipulation is too large to be experimentally tested. This is why several models of machine learning are introduced that are aimed at giving a head start in this kind of research. As mentioned earlier, findings in the study by Will et al. specifically focused at *Ophiocordyceps* are the drive for the experimental setup presented here. We want to leverage this technique further however, using the 3D-modelling and interaction software called Alphafold. (24)(25)(26) It uses googles deepmind to predict 3D structures based on amino-acid sequences. Based on these models it can predict interaction between the different proteins, which will give us a reduced list of targets. We will test these targets by combining molecular microbiology and insect genetics techniques with behavioural screening and transcriptomics.

Approach

Which Fungal Proteins bind to Rhodopsin

Since our hypothesis is that manipulation of light plays a part in the extended phenotype that we observe in infected *Camponotus floridanus* we want to focus our research on fungal proteins which specifically interact with the GPCR Rhodopsin, as explained in the previous section, since we suspect one or more that binds and blocks/inhibits the signal that this receptor produces in reaction to light. For this we will use the selection of proteins from the research of Will et al. and further reduce the number of targets based on the following criteria: secretion (since it has to reach the ant) and binding site (proteins that bind on the intracellular site are not likely to have the effect we expect). We will then separate the proteins based on binding viability as established through Alphafold 3D structure analysis, reducing the viable proteins to a smaller list. Recent Studies have shown that Alphafold is a useful tool when looking at predictions for protein interactions (24)(25)(26). We want to use the model to predict interactions of the 3D models of our fungal proteins with the 3D model of Rhodopsin of the *Camponotus floridanus*. We expect for Alphafold give us information on which of our defined group of proteins can bind rhodopsin. This will not only give us information about the binding partners, but could also give us information about the way that these proteins affect the receptor. Since we have no specialty in this field, we will ask a collaborator with expertise in Alphafold to perform this part of the research.
Secondary testing of protein interaction will be through the yeast-2-hybrid method (27)(28). A transcript of ant Rhodopsin will function as the Bait and be fused to the DNA-binding domain (DBD) while several Prey transcripts as defined by Alphafold will be fused into plasmids containing an activation domain (AD). We will transfect the prey plasmids into different Y187 yeast groups through electroporation and grow them in pairings with Y2HGold yeast cells containing the Bait-plasmids.(29) (30) For culture we will be using a dropout-media. This means that yeast cannot grow because they will be missing certain amino acids. This will make sure that only the yeast cells that allow the interaction between our plasmids will be able to grow. We will include a positive control with a known pairing. We will perform PCR on the colonies to confirm the proteins that cause the observed readout.

Functional Testing of the protein

After identifying the specific protein(s) that bind with the Rhodopsin of *Camponotus floridanus*, we will test what this protein does for the interaction between the fungi and the ant. Our hypothesis is that when we will inject the fungal proteins into the ants, part of the extended phenotype will present itself, with ants favouring higher light intensity environments. For this purpose we will synthesize the found protein(s) in the *Kluyveromyces lactis* yeast model, reason being that expression in this yeast is well documented and effective (31). We also expect better post-translation modifications when we express the proteins in another fungi. We will design new plasmids, containing a clean insert of the protein(s) with promotor and terminator sequences(i.e. without the DBD or AD domains). These will once again be transfected in yeast, however this time we will introduce the α-MF sequence, which will set up proteins to be secreted into the media.(31) This is preferred for our analysis, since in their natural host these are also secretory proteins (since they are introduced into the host) and it has been shown that this eases the production system (32). The designed plasmid will also contain a His-tag sequence for purification. To enhance the chance of proper folding, the his-tag will be attached to the proteins by a linker. (33)
We will use cobalt-based purification of protein since this delivers us the highest specificity. (34)
After purification we will make dilution series of the protein, selecting several groups of ants and injecting them in a dose-dependent matter. We would like to see if the amount of protein in the host system affects the behaviour of the ant, and might hint at a gradual process of manipulation.
We will inject the ants through micropipettes between the first and second pair of legs.(35) Previous studies have shown that this method gives the ant time to recover by closing the wound through pressure of movement of the legs. We will also have a control group of ants, which will be injected as well (but with PBS), for comparison of procedure.

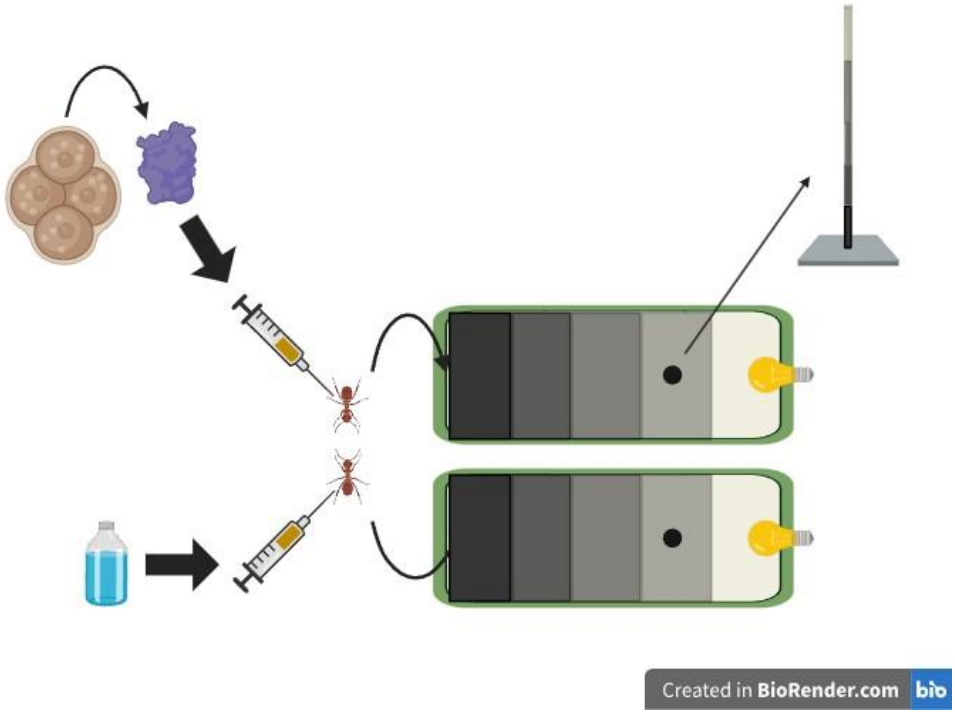

Figure 1 Test setup for behavioural read-out of fungal protein injection. Ants will be injected with either fungal protein or PBS and put into an elongated test room lighted from 1 side. The setup will be divided into five zones. The zone furthest from the lighting will be adjusted to light-levels common for ant nests and the rest of the setup will have a gradient which increases light intensity. The second setup will include a pole with a lighting gradient as well, with which the preferred height of climbing will be monitored. Ants will be monitored by camera, recording time spent in each zone.

We will record the ants for their behaviour after injection. If ants show complications from the procedure, our procedure will be to euthanize them and replace them by a new specimen. We will log the behaviour using Cow-log as our lab has previously done in research using these ants. (35)
Our next step is to categorize the different behaviours. We will set up a double-blind scoring by three observers. To lighten the load we will divide the videos between them, but three videos will be scored by all three observers, which we will use as a guideline for their personal biases. We will determine the average and instruct the observers to adjust their grading to it. To make a clear distinction in the affected individuals, we need to make a clear distinction in behaviour states. Therefore we will build an experiment setup which resembles what we theorize to happen during live infection. As stated earlier, the hypothesis is that ants will have a lessened uptake of light, moving them outside the colony and onto a branch, biting down and ultimately killed by the fungus. We will design two setups, each with an "infected" and a control group. The first setup will test only the preference for higher light environments, making rectangular habitat, with light coming from one side forming a gradient by making layers of partly transient material (figure 1). Secondly we will test the same theory in a vertical manner, making a makeshift plant with higher light intensity at the top of the stick. We test these in two different setups, because there might be other fungal proteins then just the ones binding to rhodopsin involved in the climbing behaviour. The defined behaviour will be read out in preference of light area, measured by time within certain quadrants of the test-setup. The test-setup will be of enough length to distinguish at least five quadrants, making sure there is enough variability to distinguish , since we expect the sensitivity to be greater with the higher doses injected. For the second setup we will also make a distinction in different heights of the climbing stalk. Secondly we will setup a close-up camera to distinguish between normal climbing and clamping down, even though this second behaviour is not necessarily expected. We will also be comparing ants in groups with ants that will be put in our test setups solitary, this to see if the leaving of the nest is tied to the amount of social interaction.

Specification of the affected pathways

After confirmation that our protein(s) affect ants in vivo by our behavioural studies, we will want to confirm that Rhodopsin and the pathways beneath it are specifically influenced by the introduction of the fungal proteins in the system. For this purpose we will do a RNAi experiment into live ants and see if the silencing of these pathways incur the same behaviour. We will design RNAi constructs of several different proteins. To determine which ant proteins are affected by the manipulation of rhodopsin we will inject ants with the fungal proteins again and will snap-freeze them. The time will be determined on the average time needed for behaviour to be displayed in our previous experiment. We will then perform RNA sequencing to see the changes in expression, presumably due to the interactions with rhodopsin. We will compare these results with the pathways known to be activated by rhodopsin and create constructs which specifically target these pathways to see if we can reconstitute the same effects. To administer the constructs to the ants, they will be transfected into E.coli . (36) Construct will contain a double antibiotic control for selection of successfully transfected colonies by incubating them in the respective antibiotics to kill of any interfering bacteria.(36) The bacteria will then be incubated for development of dsRNA after which they will be heat-inactivated. RNA production will be checked on agar gel with a control group containing the vector without insert. After confirmation bacteria will be introduced into ants through modified diet. (36) A control group of ants will get modified diet including bacteria as well, but the vectors contained in these bacteria will be empty besides the antibiotic control (36).

Behaviour will be quantified as stated in the previous experiment, to make the readouts of protein and RNAi comparable. After confirmation of behavioural effect, we will collect affected ants to confirm successful RNAi through RT-PCR (36) in which we will compare a reference household gene with the blocked genes to confirm abolished transcription.

Risk Assessment

Even though we have good assumptions to assume that at least one of the proteins that have been found in our previous research will bind to rhodopsin, if we get no positive results from Alphafold and our Yeast-2-Hybrid assays we would have to continue this research with a different GPCR. (13) Other parts of the research could continue following findings for these other proteins.

If the His-tag ends up impairing the interactions between receptor and our selection of fungal proteins, we will have to move to a more round-about way of isolating the proteins from our culture. We propose making columns containing rhodopsin, since after our yeast-2-hybrid we know for sure that they will bind this. This comes with the caveat that it will be less specific than our his-tag purification, due to the possibility of yeast proteins binding as well. This could be countered by adding an additional purification step, for which we propose liquid chromatography.

If secretion of the protein by yeast constitutes to changes in the protein, we might need to change the plasmids used in the production of protein to ones without the excretion sequence. Another course of action could be to take another expression system. For this we would prefer another yeast, like *Yarrowia lipolytica*, (31) but something like *E.Coli* could also be an excellent option, because this expression system is widely used.

Impact

As stated earlier the field of proteomics within the domain of zombie fungi is still relatively unexplored. Recently large scale data for specific species have been obtained, but the specific interactions and/or functions of these proteins is still not known. This research will start to give us an insight in how these species manipulate their host. This is not only applicable to *Camponotus floridanus*, since there are several other parasitic relationships in which different behaviour around light has also been described.(5)(8)(9)(17-20) One of the examples for this is *Leucochloridium paradoxum* a parasitic worm infecting snails.(37) They infest the snails eyestalk and make them less sensitive to light, so the snails are drawn outside, to be eaten by birds, the next host for the worm. Off course there are also several parasites that are known to affect the eyes of humans in different ways, like *Onchocerciasis* causing river blindness. Additionally, since Rhodopsin and other GPCR's are highly conserved within eukaryotes, (15) this might give us information into the possibility of the fungi manipulating other species. Building upon that, if we uncover the mechanisms of this light-manipulation, we might find similar proteins in other parasites which achieve the same.

Ethical Considerations

Due to the *Camponotus floridanus* not being endangered, no specific permissions are needed for the work on these animals except the rights for collection and colony upkeep previously obtained by our lab. Number of colonies and replicates needed for this experiment setup will be calculated based upon the need for statistical significance and otherwise be kept to a minimum. Colonies will be cared for in a controlled setting with access to food and water, to have minimal impact on suffering of the animals.

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