The OptoGenBox - a device for long-term optogenetics in *C. elegans*

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Optogenetics controls neural activity and behavior in living organisms through genetically targetable actuators and light. This method has revolutionized biology and medicine as it allows controlling cells with high temporal and spatial precision. Optogenetics is typically applied only at short time scales, for instance to study specific behaviors. Behavior controls systemic physiological processes. For example, arousal and sleep affect aging and health span. To study how behavior controls key physiological processes, behavioral manipulations need to occur at extended time scales. However, methods for long-term optogenetics are scarce and typically require expensive compound microscope setups. Small model animals have been instrumental in solving the mechanistic basis of medically important biological processes. We developed OptoGenBox, an affordable and simple-to-use device for long-term optogenetic manipulation of small organisms. OptoGenBox provides a controlled environment and is programmable to allow the execution of complex optogenetic manipulations over long experimental times of many days to weeks. To test our device, we investigated how optogenetically increased arousal and optogenetic sleep deprivation affect survival of arrested first larval stage *C. elegans*. We optogenetically activated the nociceptive ASH sensory neurons using ReaChR, thus triggering an escape response and increase in arousal. In addition, we optogenetically inhibited the sleep neuron RIS using ArchT, a condition known to impair sleep. Both, optogenetically increased arousal as well as optogenetic sleep deprivation reduced survival. Thus, OptoGenBox presents an affordable system to study the long-term consequences of optogenetic manipulations of key biological processes in small animals.

Keywords: Caenorhabditis elegans, *C. elegans*, optogenetics, arousal, sleep, lifespan

Introduction

Optogenetics can control many physiological processes by actively influencing biochemical reactions and manipulating neuronal activity (Fenno, Yizhar, & Deisseroth, 2011). A light-sensitive actuator can be genetically expressed in specific cells of organisms and activated by light. Different tools exist for either activation or inhibition of excitable cells. One of the most used tools are channel rhodopsins and ion pumps, which have first been discovered in algae (Nagel et al., 2002, 2003) and can now be genetically expressed in other organisms to depolarize or hyperpolarize cells upon light stimulation. Optogenetics has become widely established in different model organisms, e.g. small nematodes and flies but also mammals such as mice and monkeys (Fenno et al., 2011). However, there are still limitations that hinder the complete realization of potential of optogenetics. Especially long-term optogenetic experiments have rarely been conducted (Schultheis, Liewald, Bamberg, Nagel, & Gottschalk, 2011). In a standard experiment the neuronal manipulation only lasts for some seconds or minutes. While it is true that some reactions and neuronal signals are fast acting, to manipulate physiology in the long term, one typically has to manipulate biological processes for days or even longer. Optogenetic long-term experiments are challenging for several reasons:

(1) It is necessary to control the environment of the tested organisms.
(2) For high-throughput experiments, many different conditions should be processed in parallel.
(3) There is currently no inexpensive device available to account for 1 and 2.

Through optogenetic long-term manipulations it is possible to investigate how a specific behavior affects organisms systemically. *C. elegans* is well suited and established for optogenetic studies (Husson, Gottschalk, & Leifer, 2013; Schmitt,

Schultheis, Husson, Liewald, & Gottschalk, 2012). Many physiological processes are conserved across species and can be studied in less complex organisms such as the 1mm long nematode *C. elegans*. 83% of its genes have human homologs, allowing molecular studies that are of relevance also to human biology (Lai, Chou, Ch'ang, Liu, & Lin, 2000). With 302 neurons its nervous system is better manageable than that of other animals. Additionally, a single neuron in *C. elegans* can act similar to brain regions in mammals (Altun, Z.F. and Hall, 2011). Due to the nematode's transparency, optogenetic experiments can be conducted in a non-invasive manner (Husson et al., 2013). *C. elegans* was the first animal in which optogenetics has been established (Husson et al., 2013). However, also in *C. elegans* research the above-mentioned challenges persist. Through the development of new rhodopsins, first steps towards long-term optogenetics have been made. These newer genetic tools can be activated for minutes (Gengyo-Ando et al., 2017) or even up to 2 days (Schultheis et al., 2011). The longest optogenetic lifespan experiment to date lasted 2.5 hours (De Rosa et al., 2019). Optogenetic survival assays lasting several days or weeks have not yet been conducted in this organism.

One additional reason that explains why long-term experiments have rarely been conducted in *C. elegans* is that blue light, which is oftentimes used in optogenetic experiments, is harmful to the worms (Edwards et al., 2008; Ward, Liu, Feng, & Xu, 2008). Alternatively, optogenetic actuators have been developed that can be excited with a higher excitation wavelength, thus causing less stress to *C. elegans*. For example, the red-shifted Channel Rhodopsin (ReaChR) can be used for neuronal activation (Lin, Knutsen, Muller, Kleinfeld, & Tsien, 2013) or ArchT, which hyperpolarizes neurons by pumping out protons, for inhibition (Okazaki, Sudo, & Takagi, 2012). These genetic tools allow the use of orange light (605nm) for excitation.

Increased arousal and decreased sleep affect the survival of *C. elegans* (De Rosa et al., 2019; Wu, Masurat, Preis, & Bringmann, 2018). Many assays that promote arousal and sleep deprivation in *C. elegans* build on external stimuli such as a tapping mechanisms, the ablation of neurons or mutation (Bringmann, 2019; Driver, Lamb, Wyner, & Raizen, 2013; Hill, Mansfield, Lopez, Raizen, & Van Buskirk, 2014; Schwarz & Bringmann, 2013; Singh, Ju, Walsh, DiIorio, & Hart, 2014; Spies & Bringmann, 2018; Van Buskirk & Sternberg, 2007). Optogenetics activates or inhibits specific neurons and therefore allows the dissection of neuronal mechanisms. ASH is a nociceptor and its activation causes a reverse escape response by activating the second layer RIM interneurons and by inhibiting the sleep neuron RIS (Kaplan & Horvitz, 1993; Maluck et al., 2019). Mechanical tapping or optogenetic RIM activation, which causes a flight response and increase in arousal, shortens the lifespan of adult *C. elegans* (De Rosa et al., 2019). Depolarization of ASH causes a complex response. It activates RIM, therefore triggering release of tyramine and promotes the flight response (De Rosa et al., 2019; Maluck et al., 2019). Additionally, strong RIM activation inhibits the sleep neuron RIS which leads to sleep deprivation (Maluck et al., 2019). RIS is a single neuron that acts as the motor of sleep in *C. elegans* (Bringmann, 2018; Turek, Lewandrowski, & Bringmann, 2013). A more specific experiment for sleep deprivation, in which arousal also gets increased, is hence the inhibition of the sleep neuron RIS through optogenetics (Maluck et al., 2019; Wu et al., 2018).

To solve the problem of long-term optogenetic manipulation, we have developed the OptoGenBox, a simple-to-use device, which provides a controllable environment and allows for the execution of complex optogenetic protocols. The added material costs of less than 3500 USD makes it a lot cheaper than the use of standard microscope

set-ups. We successfully tested the OptoGenBox by optogenetically activating the sensory neuron ASH and inhibiting the sleep neuron RIS. We tested whether optogenetic activation of ASH causes a lifespan reduction in L1 arrested animals and found that it did. We then investigated what effect inhibition of RIS has on L1 arrested survival and found that it shortened survival time. Our results show that the OptoGenBox is a valuable tool for long-term optogenetic experiments in *C. elegans*, and potentially also for other small animals.

Results

A device for optogenetic long-term imaging

We developed the OptoGenBox to enable long-term optogenetic experiments in *C. elegans*. Worms were kept in a temperature-controlled environment and illuminated with orange light from the bottom (Figure 1A). For this, the OptoGenBox was built as a 70x70x90cm large device that is programmable via a touch display (Figure 1B and S1). The inside consists of a 22x22cm sized experimentation area partitioned in 13 cells (Figure 1C). Each cell can hold small plates with nematode growth medium or microfluidic chambers (Bringmann, 2011; M Turek, Besseling, & Bringmann, 2015) with a diameter of 3.5cm, and can thus fit up to 100 worms. Worm plates are placed on 4mm thick glass (B270), which was polished on the bottom (400 polish) to homogeneously distribute the LED light throughout the worm plate. 6 LEDs are distributed throughout an LED module 9mm below the glass to illuminate the worms from the bottom (Figure S2 and S3). An aluminum casing keeps external light out and creates optically isolated cells. Furthermore, the box is temperature controlled through Peltier devices and protected from external disturbances via foam and an acrylic case (Figure S4).

The researcher can easily program the optogenetic protocol through the touch screen. The system is written in Python and implemented on a Raspberry Pi (Figure S5). To start an experiment the exact cells can be selected individually for each experiment and then define the optogenetic protocol (Figure S6). The experimenter can choose how many cycles should run with how much time (hours or minutes) at light and how much time at darkness and can define the light intensity (between 2-40mW) during the light times. The temperature can be chosen for the entire OptoGenBox between 15-25°C (Figure S7). While one experiment can include up to 13 cells, individual or groups of cells can also be programmed separately to allow for parallel experiments (Figure S8 and S9). The added material costs of less than 3500 USD (Table S1) make it much more inexpensive than microscopic set-ups, which one could also use for optogenetic long-term experiments. All code and construction files are freely available at (add link here later) so that the setup of the system is straightforward for someone with technical expertise. Thus, the OptoGenbox presents an inexpensive and user-friendly tool to conduct optogenetic long-term experiments.

Optogenetic ASH activation in OptoGenBox triggers an escape response

The sensory neuron ASH is known to promote reverse escape locomotion upon different harmful stimuli (Kaplan & Horvitz, 1993; Zheng, Brockie, Mellem, Madsen, & Maricq, 1999). To test for the functionality of the box, we developed an escape essay in which we optogenetically activated ASH and tested for its effects on behavior. For this, a small plate was prepared with a small lawn of *E. coli* OP50 on one side and an opaque sticky tape on the other side (Figure 2A). Worms without any optogenetic activation were expected to mostly assemble by the food. On the contrary, after ASH activation worms were expected to not gather at the food but to either escape throughout the plate or gather in the shade, where the activation is interrupted. An optogenetic

protocol was run for one hour and the distribution of worms was counted. Indeed, an average of 80% of the control worms gathered by the food. Only around 20% of the ASH activated *C. elegans* could be counted at the food drop. This significant decrease in worms at the food drop confirms that the worms show an escape response upon ASH activation. Interestingly, worms in which ASH was optogenetically activated were not able stay in the shade. Neither the worms in which ASH was activated nor control worms were able to excape from the plate (Figure 2B). These results demonstrate the functionality of the OptoGenBox.

Increased arousal and decreased sleep by optogenetic manipulations shortens the lifespan of arrested L1 larvae

Increased arousal and sleep deprivation has been shown to shorten the lifespan in *C. elegans* (De Rosa et al., 2019; Wu et al., 2018). We wanted to test if an increase in arousal through optogenetic manipulations or inhibition of sleep can affect the survival of arrested L1 larvae. We therefore conducted experiments in which arousal gets increased through different optogenetic manipulations.

The optogenetic manipulations were achieved by treating transgenic worms carrying the optogenetic tool with ATR. Since a toxicity of ATR could not be excluded we first investigated the effects of ATR on the Wild type. Two rounds of experiments confirmed that the addition of ATR without optogenetic manipulation did not lead to a significant reduction of survival (Figure 3A and S10A). Hence, any lifespan phenotypes in our optogenetic experiments can be attributed to the optogenetic manipulations and not the treatment with ATR.

To test for survival phenotypes upon increased arousal, we conducted two experiments, a first experiment in which optogenetic activation of a nociceptive neuron

causes an escape response and increases arousal and a second experiment in which optogenetic inhibition of a sleep neuron causes sleep deprivation.

For the optogenetic activation experiment we expressed ReaChR in the pair of ASH neurons. All-trans retinal (ATR) was present throughout the L1 arrest lifespan to ensure functionality of the optogenetic tool. Control worms were used that carried the ReaChR transgene but did not receive ATR. In both rounds of the experiment, animals in which ASH was activated died significantly earlier than control worms (Figure 3B, Figure S10B).

Next, we tested how sleep deprivation caused by the inhibition of the sleep neuron RIS affects survival in L1 arrest. We expressed ArchT under the *flp-11*promoter so that it was specifically expressed in RIS and all-trans retinal was supplemented (Wu et al., 2018). Again, control worms for comparison did not receive ATR treatment. Optogenetic sleep deprivation led to a small but significant reduction of survival in arrested L1 animals by 8.3 % (Figure 3C and S10C).

Discussion

Here we developed the OptoGenBox as a device for optogenetic long-term experiments. OptoGenBox combines a controlled environment and allows for parallel processing of many experiments for small animal models. With material costs of less than 3500 USD it is rather inexpensive. Experiments can last up to several weeks.

We could demonstrate that different optogenetic manipulations that increase arousal and inhibit sleep have a detrimental effect on *C. elegans*. The activation of the nociceptor ASH led to a reduced survival of L1 arrest. While ASH activation also leads to an inhibition of the sleep neuron RIS, the lifespan shortage of ASH activated animals cannot solely be accounted for by sleep inhibition (Maluck et al., 2019). More likely, the reduced survival upon ASH activation is caused by the inhibition of cytoprotective

mechanisms through the activation of RIM and release of tyramine as has been previously described (De Rosa et al., 2019).

The optogenetic inhibition of RIS presents a very specific and therefore suitable experiment to investigate the effects of sleep deprivation on *C. elegans*. The shortened survival upon RIS inhibition confirms that sleep plays an essential role in arrested L1 worms as has been previously demonstrated with *aptf-1(gk794)* mutants in which RIS is not functional and with worms in which RIS was genetically ablated. In experiments with worms in which RIS function was impaired, it was shown that sleep counteracts aging phenotypes (Wu et al., 2018). It would be interesting to see how aging phenotypes progress when RIS gets inhibited optogenetically. Additionally, how exactly sleep counteracts aging and causes premature death needs further investigation.

Conclusions

With the newly developed device for optogenetic long-term experiments, the OptoGenBox, we have mostly investigated how an increase of arousal affects survival in L1 arrest. However, many other questions could be answered with our device. Optogenetics is a method that cannot only be utilized for depolarizing or hyperpolarizing neurons but also any other type of cell such as epidermal or muscle cells. Silencing of body wall muscles for example leads to an inhibition of feeding (Takahashi & Takagi, 2017) and photoablation of epidermal cells causes paralysis in *C. elegans* (Xu & Chisholm, 2016). The OptoGenBox should allow for many optogenetic long-term experiments in *C. elegans* and potentially also other small animals. Long-term optogenetics should thus help understand how behavior affects systemic physiology in the long term.

Methods

Development of the OptoGenBox

Further information is included in the supplement.

C. elegans maintenance

Worms were grown at 20°C on Nematode Growth Medium (NGM) plates. The plates were seeded with *E. coli* OP50, which served as food for the worms (Brenner, 1974). The following strains were used for this study:

HBR974	*goeIs232(psra-6::ReaChr::mKate2-unc-54-3'utr, unc-119(+))*
HBR1463	*goeIs307(pflp-11::ArchT::SL2mKate2-unc-54-3'utr,unc-119(+))*
N2	Wild type(Bristol) (Brenner, 1974)

Escape Assay

Late L4 stage worms were picked onto NGM plates with 0.2 mM all-*trans* retinal (ATR, Sigma Aldrich). Control late L4 stage worms were picked onto NGM plates without ATR. 9.6 cm^2 large NGM plates were prepared for the experiment by placing a 1 cm^2 opaque tape on the bottom of one side of the plate and a drop of *E. coli* OP50 on the other side (Figure 2A). After 4 hours, 10 young adult worms were picked into the food drop of the experimental plate for each trial.

The experimental plates were then placed in the OptoGenBox and stimulated with 10mW orange light for 1 hour at 20°C. After one hour the plates were removed from the OptoGenBox and the distribution of worms on the plates was counted.

Lifespan assay

It was shown before that sleep is important for the survival of *C. elegans* by

counteracting aging phenotypes. However, non-sleeping *aptf-1(gk974)* mutants only have a reduced lifespan when worms starve upon hatching and arrest in the first larval stage (L1 arrest) and not when they are adults (Wu et al., 2018). For this reason, we conducted our experiments with L1 arrested animals.

Worms were kept in microfluidic devices as previously described (Bringmann, 2011; M Turek et al., 2015). A PDMS mold was used as a stamp to cast 110x110x10µm cuboids into a hydrogel. The hydrogel consisted of 3% agarose dissolved in S-Basal (Stiernagle, 2006). Eggs were transferred from a growing plate to a plate without food and then picked into chambers without transferring food. Between 29 and 45 worms were in one microfluidic device housed in individual chambers.

For optogenetic activation or inhibition, chambers were replenished with 10µl of 10mM all-trans-retinal (ATR, Sigma Aldrich) all 3-4days. Control chambers did not receive ATR. To avoid fungal contamination, 20µl of 10µg/ml nystatin was pipetted to each chamber 2-4 times throughout the lifespan. Additionally, 20µl of sterile water was added all 2 days until day 15 of the lifespan and then each day to counteract the agarose drying out over time. In the beginning of the lifespan experiment, worms were counted every second day, in the later stages of the survival assay they were counted every day. A worm was counted as dead if it didn't move for 2 min under stimulation with a blue light LED.

The worms were placed in the OptoGenBox and illuminated with 10mW of 605nm LEDs for 11h. This was followed by 1h of darkness. This protocol was repeated until all worms were dead. The temperature of the incubator was set to 20°C.

Statistics

Sample sizes were determined empirically based on previous studies. The researcher was not blinded since the addition of ATR is easily detectable. Conditions in the escape

assay were compared with the Kolmogorov-Smirnov-Test. For the lifespan assays the day was determined when the shorter-lived condition reached 50% survival. Data of both conditions were compared on that day with Fisher's Exact Test.

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Author Contributions

IB and FJ designed the OptoGenBox. IB designed, performed and analyzed the experiments and wrote the manuscript. FJ built the hardware of the OptoGenBox. PS programmed the software of the OptoGenBox. HB acquired funding, conceived the project, supervised the work, and edited the manuscript.

Disclosure of Interest

The authors declare that they have no competing interest.

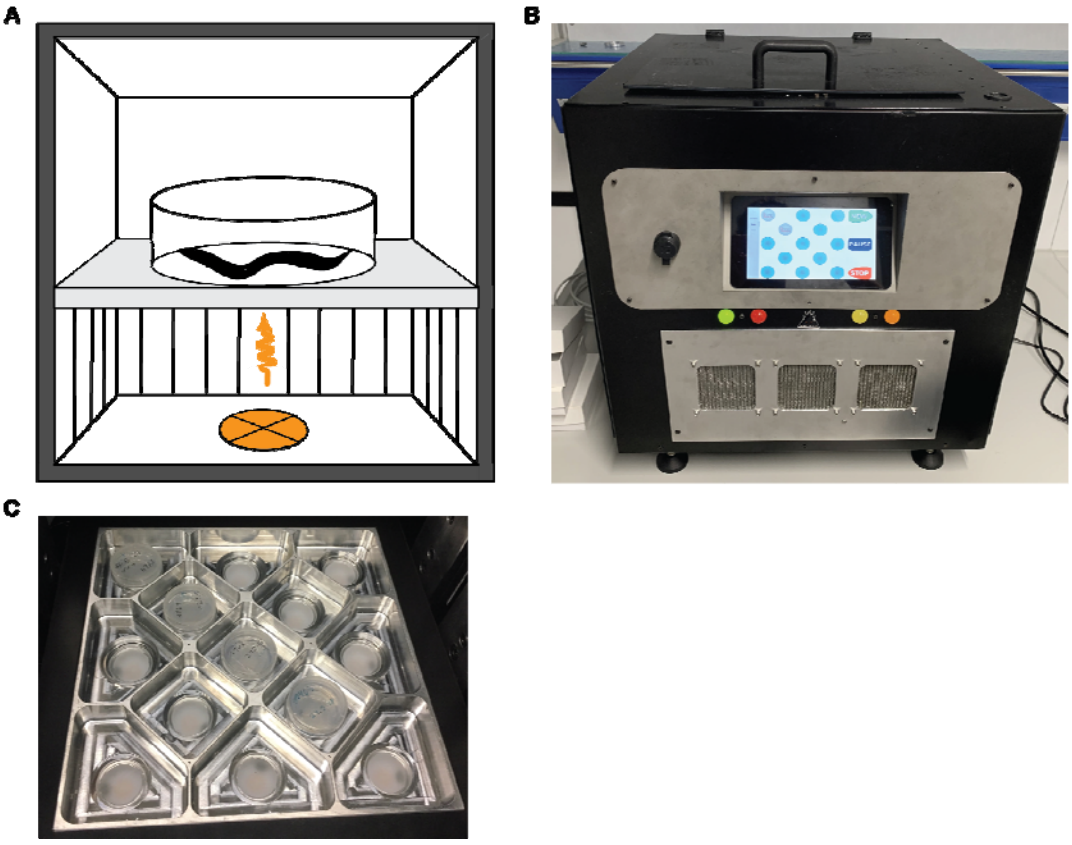

Figure 1. The OptoGenBox is a device for long-term optogenetic experiments in *C. elegans*.

A) Worms are kept in a controlled environment and illuminated with orange light.

B) The outside of the OptoGenBox.

C) The inside of the OptoGenBox is comprised of 13 groupable or separately programmable cells.

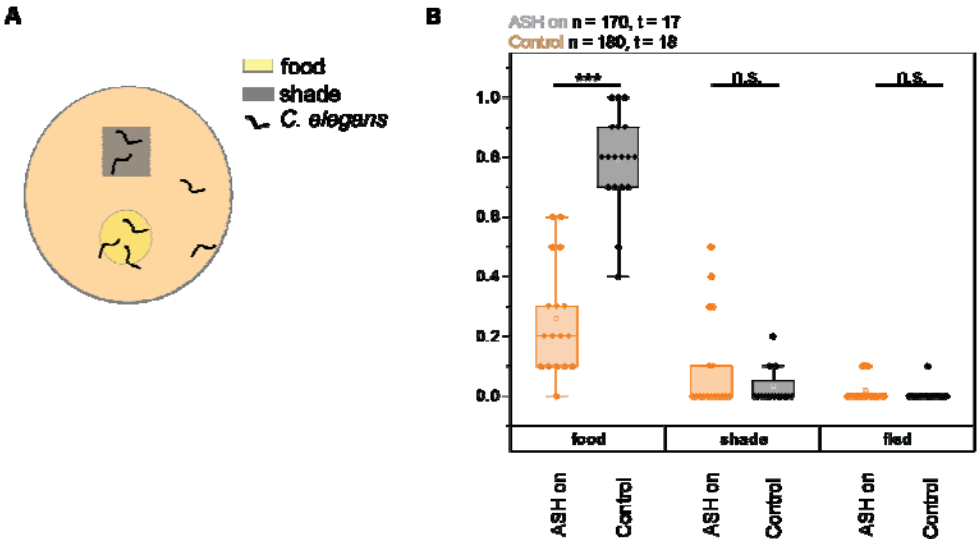

Figure 2. ASH activation in the OptoGenBox caused an escape response.

A) Preparation of the experimental plate.

B) After ASH activation worms did not stay on the food droop but distribute throughout the plate. ***p<0.001, Kolmogorov-Smirnov-Test.

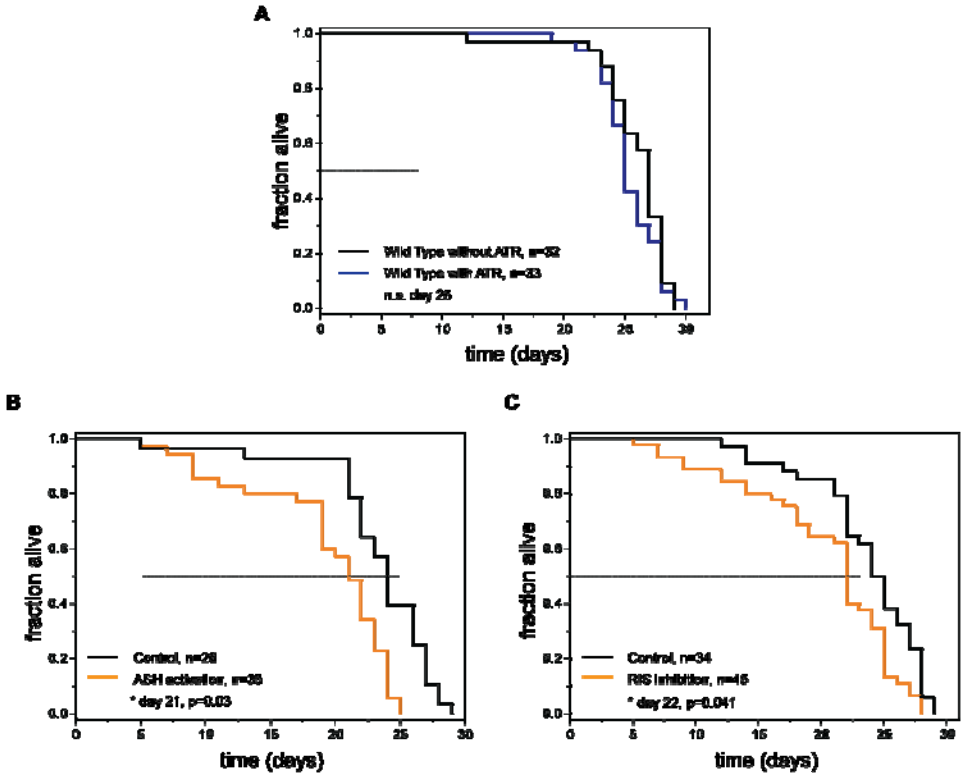

Figure 3. An increase in arousal and sleep deprivation reduces the lifespan of arrested L1 *C. elegans*.

A) All-trans retinal (ATR) did not affect survival of arrested L1 larvae. n.s. $p>0.05$, significance shown from day 25, Fisher's Exact Test.

B) ASH activation causes a reduction in lifespan. *$p<0.05$, significance shown from day 21, Fisher's Exact Test.

B) RIS inhibition causes a reduction in lifespan. *$p<0.05$, significance shown from day 22, Fisher's Exact Test.

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