Investigating the Role of Glia in Sleep and Sensory Processing through *lin-42* PERIOD homolog in *C. elegans*

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ABSTRACT

The relationship between glia, sleep, and sensory processing has not previously been well studied, but the use of *Caenorhabditis elegans* (*C. elegans*) as a model organism has made such research possible. We utilized a reverse genetic screen to identify genes of interest expressed in *C. elegans* glia by using an online gene atlas, WormGlia.org. We focus here on the *C. elegans* circadian clock gene homolog *lin-42*, which has been described to impact sleep. However, it is unclear whether glia contribute to this defect. We used sensory processing assays to make a link between sleep and sensory processing, as *C. elegans* glia are known to impact sensory processing. To analyze the animals' sleep behavior, we additionally compared two high throughput machine learning models and two *C. elegans* arenas to determine what would give us the best output data.

INTRODUCTION

Sleep disorders have previously been found to be associated with autism in humans, cementing the relationship between sleep disturbance and sensory processing disorders. In autism, indicative sleep abnormalities including sleep anxiety, night awakening, and short sleep duration have been found to be related to sensory integration and processing[1]. Glial cells are non-neuronal cells involved in many aspects of normal brain function. Glia are active participants in neurodevelopment, synaptic modulation, and homeostasis including clearing waste and

regulating synaptic function and pruning[2]. Given their involvement in neural communication, the study of glia could offer insights into how sleep disruptions and sensory processing difficulties are related.

In mammals, it has been found that a specific type of glia, called astrocytes, can impact sleep[3]. However, further study is very difficult in laboratory animals such as drosophila (fruit flies), zebrafish, and mice where it is difficult to simultaneously study cell-cell interactions at a cellular and behavioral level and also lack a consistent, well-mapped connectome. Instead, the model organism *C. elegans* is used because of its simple nervous system consisting of only 306 neurons and 56 glia, its powerful genetics that can reproducibly target individual cells or groups of cells, its invariant developmental lineages and neuron-glia contacts, its well-mapped connectome, and the ability to zoom seamlessly from observing single genes or cells to whole animal behaviors.

C. elegans experience two types of sleep: developmentally timed sleep (DTS) and stress-induced sleep (SIS). We focus on DTS in our study of sleep in *C. elegans* because its features, such as behavioral immobility, reduced responsiveness to external stimuli, and homeostatic regulation, closely resemble canonical sleep in mammals and *Drosophilia*[4,5,6]. Furthermore, *C. elegans* shares homologs of circadian clock genes found in mammals and *Drosophila*, including PERIOD, ROR, and TIMELESS. Mutations in the PERIOD gene homolog *lin-42* cause sleep disruption, including aberrant timing of molting and DTS behavior.[7,8].

While we know that glia impact sleep in other organisms, we know little about how glia in *C. elegans* impact sleep. A recent study found that the Cephalic Sheath (CEPsh) glia, which form the nerve ring of *C. elegans* and ensheath the synapses of many sensory neurons, impacts locomotion of the worm during sleep through its connections with the sleep-related neurons

ALA and the interneuron AVE[9]. Dysfunction in the glia can cause a lack of movement before sleep periods, uncharacteristically prolonged sleep, and short pauses in movement that are not aligned with the molting of the worm[10]. This discovery provides further evidence for similarities in the associations between glia and sleep in mammals and in *C. elegans*.

Studies exploring the role of *C. elegans* glia in sensory processing have shown that glia play a bigger role in the process than originally assumed. The amphid sheath (AMsh) glia which is located at the nose-tip of the worm ensheaths the AFD sensory neuron and is known to independently balance different sensory inputs (temperature and smell) and how they are communicated to different neurons as well as play a vital, autonomous role alongside, instead of assisting, the ASH neuron in olfactory processes such as the detection of repulsive odorants[11,12]. This brings into question whether glial cells are similarly vital to sleep and sleep regulation in *C. elegans* and suggests that *C. elegans* glia modulate sleep through their role in regulating sensory processing functions.

RESULTS

Sensory processing assays

To assess if *lin-42* mutants with sleep disturbances also exhibit sensory processing defects, we performed a set of established thermotaxis, chemotaxis, and mechanosensory assays. *C. elegans* are known to thermotax to their temperature of cultivation[13]. We performed thermotaxis on two *lin-42* mutant alleles ok2385 and n1089[14] and found that both *lin-42* mutants had thermotaxis behavior that differed from that of wild-type worms (Fig. 1B), where *lin-42* mutants did not properly thermotax to their cultivation temperature of 15°C as compared to wild-type *C. elegans.*

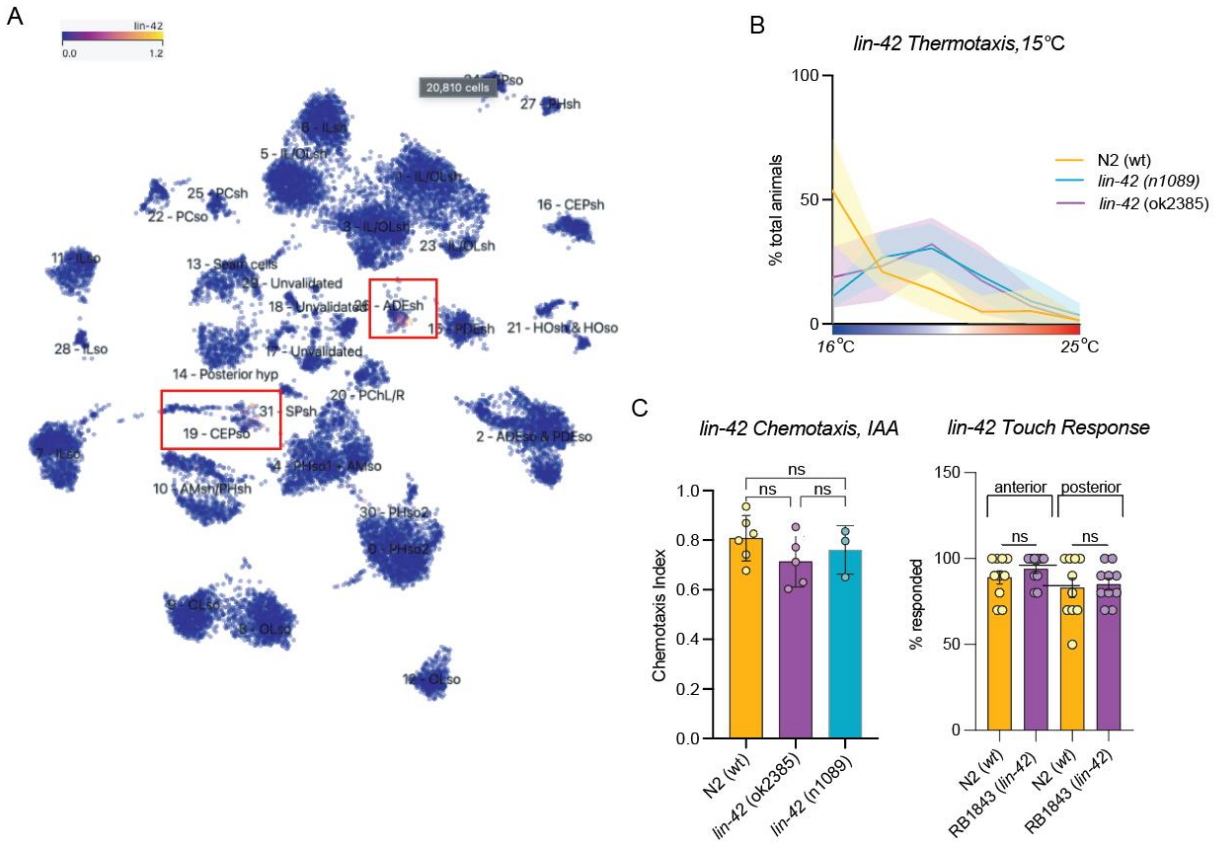

Figure 1. Sensory processing assay in *lin-42* mutants. A) expression of *lin-42* in glia from wormglia.org. Heat-map in upper right corner indicates expression level for cells within individual glial clusters. Boxed red areas highlight *C. elegans* glia with highest lin-42 expression B) animals with *lin-42* mutant alleles cultivated at 15°C thermotax improperly compared to wild-type animals. C) *lin-42* mutants do not show defects with isoamyl alcohol chemotaxis (D) *lin-42(ok2385)* mutants do not show mechanosensory defects.

Next, we performed a chemotaxis assay with the same *lin-42* mutants (Fig. 1C). C. *elegans* have well-described chemotaxis behavior and navigate to or away from specific olfactory and gustatory cues. Neither *lin-42* alleles were found to have chemosensory defects toward a preferred odorant, Isoamyl alcohol. Chemotaxis indices of ok2385 and n1089 mutant alleles were not statistically different from wild-type ($p = 0.2387$, $p = 0.7729$, respectively, using

one-way ANOVA with Tukey's multiple comparisons test). Furthermore, no statistically significant difference was observed when comparing both alleles (p=0.7896).

Lastly, we performed a commonly used mechanosensory assay, in which the anterior and posterior end of the worm are stroked with an eyelash pick (Fig. 1D). Our results showed that *lin-42* (ok2385) mutants did not display any significant mechanosensory defects, where there was no difference between the percent of mutant and wild type animals that responded to touch applied to the animals anterior (p=0.6204) or posterior end (p=0.9252, two-way ANOVA)[15].

Sleep assays

In order to verify reported *lin-42* sleep disruptions[16] and to create a sleep analysis pipeline for future mutants, we tested several different imaging and analysis methods for assaying *C. elegans* sleep. The first method we used, called WorMotel, involves creating a PDMS mold with small wells filled with agar and OP50, allowing simultaneous imaging of 48 individual worms. The resulting concave surface of the agar in each of the wells and aggregation of OP50 near the walls of the well incentivized the worms to explore and remain close to the edges of each well. Furthermore, copper (II) sulfate ($CuSO_4$), used to prevent the worms from moving into neighboring wells, seeped into the agar and appeared to make the worms sick and immobile during the imaging session. Worms aggregating in the low-contrast areas very close to the edges of the wells resulted in unusable training data during subsequent analysis, therefore, we explored other options for recording *C. elegans* sleep behavior. For the following method, we designed a custom PDMS plate for imaging. This plate mimicked WorMotel with modifications such as a small main well to hold more than one worm and a wider moat surrounding the main arena containing $CuSO_4$. These modifications allowed us to circumvent the issues of containing individual worms and uneven agar that were observed with WorMotel. We found that a chamber

with a shallow main well filled completely with agar features eliminated imperfections or concavity in the surface of the gel. This reduced the clumping of worms during imaging and improved the model's ability to train based on this dataset, and a higher volume of surrounding $CuSO_4$ deterred the escape of the worms without impacting their apparent health.

We next tested two different analysis methods to identify worm sleep. The first model we tested, Social Leap Estimates Animal Poses (SLEAP) is an open-source deep learning algorithm that is used to track multiple animals in an arena[17]. Predictions utilizing the SLEAP model failed to improve significantly after four human-in-the-loop training cycles and 81 complete frames of training data. The minimal improvement between the third and fourth training cycles was negligible when compared to the success of using WormLab on equal quality video. The model consistently identified about 96% of worm instances, but the pose predictions remained inaccurate and inconsistent. In contrast, WormLab analysis software using video taken on the DMK 33GP031 33G Series camera consistently correctly identified and tracked all worms on the plate. Our first trial of using WormLab for analysis on video taken using the WormLab-specific imaging camera did not produce viable results due to the increased appearance of imperfections in the agar with reverse contrast.

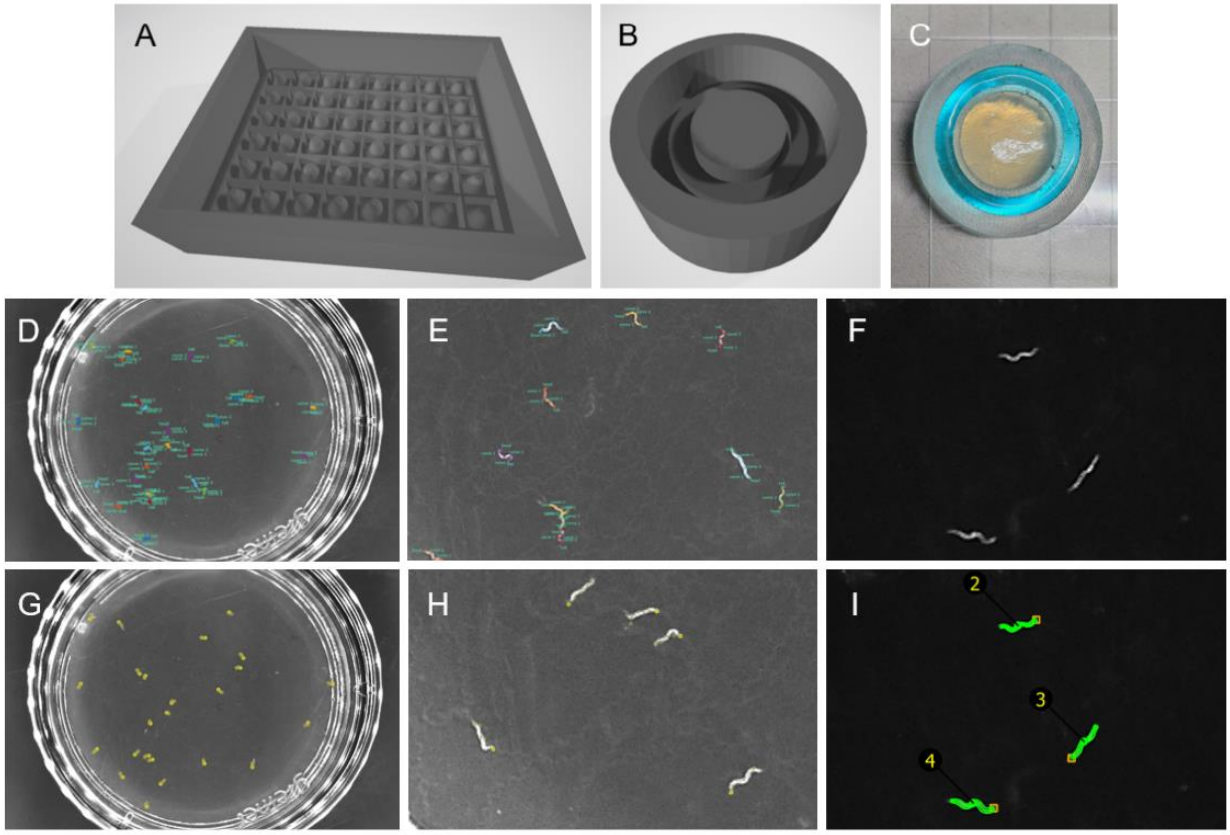

Figure 2. Analysis of *C. elegans* sleep using a custom-designed chamber and multiple analysis methods. A) WorMotel negative mold 3D rendering. B) Custom arena negative mold 3D rendering. C) Custom arena setup containing agar, worms, and $CuSO_4$ moat (turquoise). D, E) example training frame in SLEAP model. F) Example training frame in WormLab. G, H) Example test frame with labeled instances in SLEAP model after 4 training cycles. I) Example test frame with labeled instances in WormLab model

METHODS

Reverse genetic screen

For the reverse genetic screen, we selected genes of interest that are known to impact sleep in *C. elegans*, with a focus on *C. elegans* circadian clock gene homologs. Using wormglia.org, a public atlas showing the expression of different genes throughout *C. elegans* glia[18], we determined if each gene had known glial expression (Fig. 1A). We chose to focus on

the circadian clock PERIOD homolog, *lin-42,* as it has known defects in its sleep cycle, but it does not have described function in glia or a role in sensory processing[19]. We obtained two different mutant alleles with sleep defects, *lin-42* (ok2385) and *lin-42* (n1089) from previously described studies from the *C. elegans* Genetics Center (CGC)[20].

Sensory processing assays

Thermotaxis was used to assay the temperature-sensing ability of the worms on a temperature gradient between 15°C and 26°C, as previously described[21]. Gravid adults were washed using M9 from a plate into a bleaching solution (12% bleach, 5M KOH solution in M9) until the majority of eggs were released from the adults before they were washed 3x using M9 buffer and spun down at 1000 rpm for 1 min. Once spun down and excess M9 was removed, the remaining worms were plated on fresh agar plates. *Lin-42* (ok2385, n1089) day 1 adults were hand-picked due to their inconsistent development times, resulting in worms at different development stages on a plate. Day 1 adults were spun down, removing M9, before ~150 were plated at the center of a thermotaxis plate containing 12mL of NGM agar, prepared between 2 and 7 days of experiment[22]. Thermotaxis plates were placed on the temperature gradient with 7mL of glycerol on its bottom for increased thermal conductivity for 45 minutes. At the end of the assay, plates were inverted over chloroform to immobilize the worms. Using the 6x6 gridded thermotaxis plates as a guide, the percentage of animals in each temperature column bin, excluding the middle two rows, was calculated and plotted across the temperature gradient (Fig. 1A)[23].

Chemotaxis assays were performed to evaluate the olfactory abilities of the worms. After tracing counting circles for the odorant and control at each side of behavior plates, ~200 synchronized day 1 adult *lin-42* mutants were picked from OP50 plate, washed 3x using the

assay buffer, and plated using a glass pipette. 1µL 1M sodium azide was pipetted into the center of each odorant circle to paralyze worms that reached the odorant. The odorant circle contained 1µL 1:100 isoamyl alcohol diluted in ethanol, an attractant for wild-type animals, and the control circle contained 1µL ethanol. At the end of 1 hour covered by a box, the plates were inverted over chloroform to immobilize the animals and allow for counting of worms inside and outside both circles (preference vs. no preference). The chemotaxis index was calculated using the formula:

$$chemotaxis\ index\ = \frac{(no.animals\ at\ odorant - no.animals\ at\ control)}{N}$$

$$where\ N\ =\ total\ no.of\ animals\ at\ odorant, control, and\ no\ preference$$

Mechanosensory assays involve using an eyebrow hair pick and gently brushing the worm just behind the pharynx for an anterior touch response and before the anus for a posterior touch response when the worm is isolated on a clean plate without food[24]. 10 worms were assayed with ten stimuli each, alternating anterior and posterior touch responses. For both responses, the worm is said to have normal sensation if it pauses movement or reverses direction when touched.

Imaging and Sleep Arenas

We used recorded videos from two different cameras to obtain video data of worm plates. We compared image quality from a Basler ACE CMOS camera and lens used alongside IC Capture software to a DMK 33GP031 33G Series camera (associated with WormLab Software), both with red LED illumination. A polydimethylsiloxane (PDMS) WorMotel device consisting of wells for individual worms filled with agar and OP50 and channels (moats) separating the

wells containing 100 mM copper sulfate ($CuSO_4$), an aversive substance to discourage worms from leaving their wells and to prevent escaped worms from entering other wells[25]. The WorMotel plate was constructed utilizing a PDMS solution of 9g PDMS and 1g curing solution which was thoroughly combined and centrifuged for 5 minutes. After PDMS was poured into the 3D-printed negative plate mold, 1 hour in a vacuum chamber at 25 psi removed any bubbles before the plate was allowed to cure completely. Arena was carefully removed from the mold and plasma-treated to facilitate fluid flow.

A custom plate utilized a similar well and moat while reducing the imaging area. A circular well with a radius of 1 cm and depth of 0.5 cm was surrounded by a moat depth of 0.25 cm and width of 0.25 cm. A PDMS solution identical to that used for the WorMotel was used to construct the area using a 3D-printed negative mold. Once the resulting arena was sterilized with EtOH, 2 mL of NGM agar filled the main well to 0.5 mm from the brim, topped by a thin layer of OP50, and ~1 mL of 100 mM $CuSO_4$ filled the outer concentric well. Reducing the necessary field of view from that required for other plates or the worm motel allows higher resolution imaging and constrains worms to the visible area toward the center of the plate.

Sleep Analysis

To determine if other mutants of interest have sleep defects, a high throughput method of analyzing and imaging worms to quantify their sleep patterns is necessary. SLEAP, an open-source deep learning system for multi-animal pose tracking, was initially utilized to track and quantify the position of worms[26]. The program comes with a one-line installation, an easy-to-use GUI, and options for human-in-the-loop processing. It is compatible with both top-down and bottom-up identification and has both pre-trained and customizable architecture for the involved

neural networks. It was installed through the Miniconda, a lightweight Anaconda installer containing only Conda, Python, and necessary packages.

WormLab imaging software uses contrast-based tracking and determines a midline for each individual worm instance. Video was recorded on the DMK 33GP031 33G Series camera. using the custom plate design resulting in accurate identification and tracking of worms. Utilizing a lower frame rate of 3.6 fps, restricting tracking area to the area of agar, and using 10 worms on a 2cm plate increased accuracy and reduced the analysis time. The training of the model and resulting analysis was less human-in-the-loop than with SLEAP and involved only pointing out instances of worms on 1-2 frames. After analysis, unwanted instances could be manually deleted.

DISCUSSION

C. elegans glia have previously described roles in regulating the ability of the animal to respond to sensory stimuli[27,28,29]. Because *C. elegans* sleep retains the same characteristics of sleep in other model organisms, and because of the known role of glia in sensory processing, we hypothesized that glia could impact sleep by impacting the animal's sensory processing circuits. This association implies that sensory processing is necessary for functional DTS. We found that *lin-42* mutant alleles displayed thermotaxis deficits, but no change to chemotaxis or mechanosensory behavior compared to wild-type animals. Therefore, *lin-42* is tied more closely to thermosensation and temporal regulation. This suggests that *lin-42* is responsible for connecting external cues such as temperature to sleep patterns, behavior, and molt stages, especially during developmental stages.

WormLab was more successful at identifying and quantifying the position of *C. elegans* worms due to the process of the machine learning model. Unlike SLEAP, which relies on nodes to create a skeleton, a method that works well for animals with a very clear structure and skeletal body parts (flies, rodents, etc.), WormLab begins by identifying the center of instances and creating a midline for each instance on each frame. This midline can then be separated into a user-defined number of nodes. Since the midline method does not follow as strict of a skeletal structure, it is better suited to constantly moving and changing animals such as worms.

FUTURE DIRECTIONS

Our results show that *lin-42* mutants exhibit sensory processing defects in addition to existing sleep and developmental defects previously described[7]. To further examine the connection between sleep, sensory processing, and glia in *lin-42* mutants, we planned to first confirm *lin-42* expression in glia by imaging *lin-42*:GFP reporter animals co-labeled with a pan-glial maker and assessing the overlap of the two fluorophores. Our next steps once confirming expression in glia would be to perform cell-specific rescue experiments, in which we would generate plasmid DNA containing the functional segments of our gene of interest, *lin-42,* re-expressed only in subsets of worm glia. Our construct would insert a functional copy of the gene driven by the promoter for the AMsh glial cell, as it has a known role in altering thermotaxis behavior through its interaction with the AFD thermosensory neuron. We would then re-examine their behavior in the thermotaxis assay to see if rescuing *lin-42* in the AMsh glia would reverse our observed thermotaxis phenotype. WormLab tracking and subsequent analysis would then be used to verify previously reported sleep defects[30]. Lastly, based on the glial expression of *lin-42* from wormglia.org, we predicted there to be likely mechanosensory defects due to the high levels of expression in the ADEsh glia, which is associated with mechanosensory neurons.

Future mechanosensory assays can be performed to target the function of the ADEsh and midbody mechanosensory neurons more specifically.

We tested several methods for recording and analysis and found that WormLab midline analysis can be used to create segmented quantification of worm position over time. For future recordings, this data can then be parsed and analyzed to determine the average time worms spend in developmentally timed sleep stages by identifying worms not moving in the sleep stance. Differences in sleep data between future mutants of interest and wild-type strains can be confirmed to help narrow down additional genes that impact sleep, following a similar pipeline for our assessment of *lin-42*.

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