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Evidence for Secretion of a Netrin-1-like Protein by *Tetrahymena thermophila*

Victoria E. Ames

Cedarville University, victoriaames@cedarville.edu

Grant Hooper

Cedarville University, granthooper@cedarvill.edu

Aubrey J. Juris

Cedarville University, aubreyjjuris@cedarville.edu

Cole Knox

Cedarville University, coleknox@cedarville.edu

Jack Lightbody

Cedarville University, jacklightbody@cedarville.edu

See next page for additional authors

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Authors

Victoria E. Ames, Grant Hooper, Aubrey J. Juris, Cole Knox, Jack Lightbody, Alexa C. Manthei, Jacob P. Olejarczyk, Benjamin D. Swenor, Amiah Warder, Emily B. Weindorf, Taylor L. Vander Plas, and Heather G. Kuruvilla

Evidence for Secretion of a Netrin-1-like Protein by *Tetrahymena thermophila*

Victoria Ames, Grant Hooper, Aubrey Juris, Cole Knox, Jack Lightbody, Alexa Manthei, Jacob Olejarczyk, Benjamin Swenor, Amiah Warder, Emily Weindorf, Taylor Vander Plas, Heather Kuruville

Department of Science and Mathematics, Cedarville University, Cedarville, OH 45314 USA

Abstract

Netrin-1 is a pleiotropic signaling molecule with targets in many mammalian cell types. Though first characterized as a chemotactic signal involved in neuronal guidance during development, netrin-1 has since been found to have a regulatory role in angiogenesis, and is also used as a biomarker in certain cancers.

Tetrahymena thermophila are free-living protists that rely on chemotactic signals to find food, as well as to escape predators. Chemoattractants cause the cells to swim faster in the forward direction, while chemorepellents cause ciliary reversal, resulting in movement of the cell away from the noxious stimulus. We have previously found that netrin-1 is a chemorepellent in *T. thermophila*. More recently, we have detected netrin-1 by ELISA in both whole cell extract and secreted protein samples obtained from *T. thermophila*. In addition, we have immunolocalized netrin-1 staining to the cytosol of *T. thermophila* using an anti-netrin-1 antibody. Western blots using an anti-netrin-1 antibody reveal two proteins, approximately 60 and 120 kD, which are present in whole cell extract obtained from *T. thermophila*. Further experimentation is needed to determine the physiological role of this protein in *T. thermophila*.

Introduction

The vertebrate signaling protein, netrin-1, is a chemotactic signal that appears to be involved in neuronal development as well as in angiogenesis. Depending upon the neuronal cell type and the netrin-1 gradient, this protein may act as a chemoattractant, encouraging neuronal growth cone formation, or as a chemorepellent, causing growth cone collapse. Netrin-1 is also an inhibitor of leukocyte chemotaxis. Netrin signals through several different receptors within vertebrate systems, including DCC, UNC5B, and CD146, all of which use different signal transduction pathways and have divergent physiological consequences.

Tetrahymena thermophila are free-living ciliated protozoans which are often used as model systems for chemosensory behavior. These organisms respond to chemoattractants by increasing their swimming rate. In response to chemorepellents, *Tetrahymena* reverse their cilia, causing them to swim in a jerky, backward motion which allows them to reorient with respect to the repellent stimulus. We have previously found that netrin-1 peptide is a chemorepellent in this organism. We then hypothesized that *Tetrahymena* may be secreting a similar compound, allowing them to use netrin as an autocrine or paracrine signal. In our current study, we used a polyclonal antibody against netrin-1 to do ELISA, immunofluorescence, and Western blotting, to determine whether *Tetrahymena* are making a similar protein. Our results indicate that *Tetrahymena* synthesize and secrete a netrin-1-like protein which may be used in autocrine or paracrine signaling.

Materials & Methods

ELISA

1-day old cultures of *T. thermophila* were washed in behavioral buffer consisting of 1mM Tris, 0.5 mM MOPS, 50 μ M CaCl₂, pH 7.0. 5 ml of culture was concentrated into 1 ml of buffer. For secreted proteins, cells were incubated at room temperature for one hour. The cells were then pelleted by centrifugation and the supernatant was saved as our secreted protein sample. For the whole cell extract, cells were incubated on ice in RIPA lysis buffer for one hour in the presence of protease inhibitor cocktail. Both extracts were used in ELISA, using goat anti-netrin-1 IgG as the primary antibody and rabbit-anti-goat IgG, HRP conjugate, as the secondary antibody.

Immunofluorescence

Immunofluorescence was carried out using a modified protocol obtained from cell signal.com. Briefly, cells were washed twice in PBS, reconstituted in 3.7% formaldehyde in PBS, and allowed to fix for 15 min at room temperature. After fixation, cells were rinsed three times in PBS before being blocked in blocking buffer for 60 minutes. After washing off blocking buffer, cells were incubated overnight at room temperature in primary antibody at a dilution of 1:100. After rinsing three times in PBS, cells were incubated in fluorochrome-containing secondary antibody for 1–2 hours at room temperature in the dark. Cells were then rinsed three times in PBS. Ten microliters of cell suspension was then applied to a slide and mixed with one drop of DAPI. Cell suspension was then covered with a coverslip and observed under a fluorescence microscope at 400X.

Western Blotting

Protein extracts were prepared as for ELISA above. Extracts were run on a 10% SDS-PAGE. Gels were transferred to a PVDF membrane, and Western blots were performed using a 1:1000 dilution of goat anti-netrin-1 IgG as the primary antibody and a 1:5000 dilution rabbit-anti-goat IgG, alkaline phosphatase conjugate, as the secondary antibody. NBT substrate was used to show alkaline phosphatase activity.

Results

Table 1. ELISA shows evidence of netrin-1 in both whole cell extracts and secreted protein obtained from *Tetrahymena thermophila*. The ratio of netrin-1 reactivity to total protein was much higher in secreted protein fractions.

Protein Extract	Total Protein Concentration	Netrin-1 Concentration
Whole Cell Extract	114 mg/ml	~ 0.1 μ M
Secreted Protein	0.29 mg/ml	~ 0.1 μ M

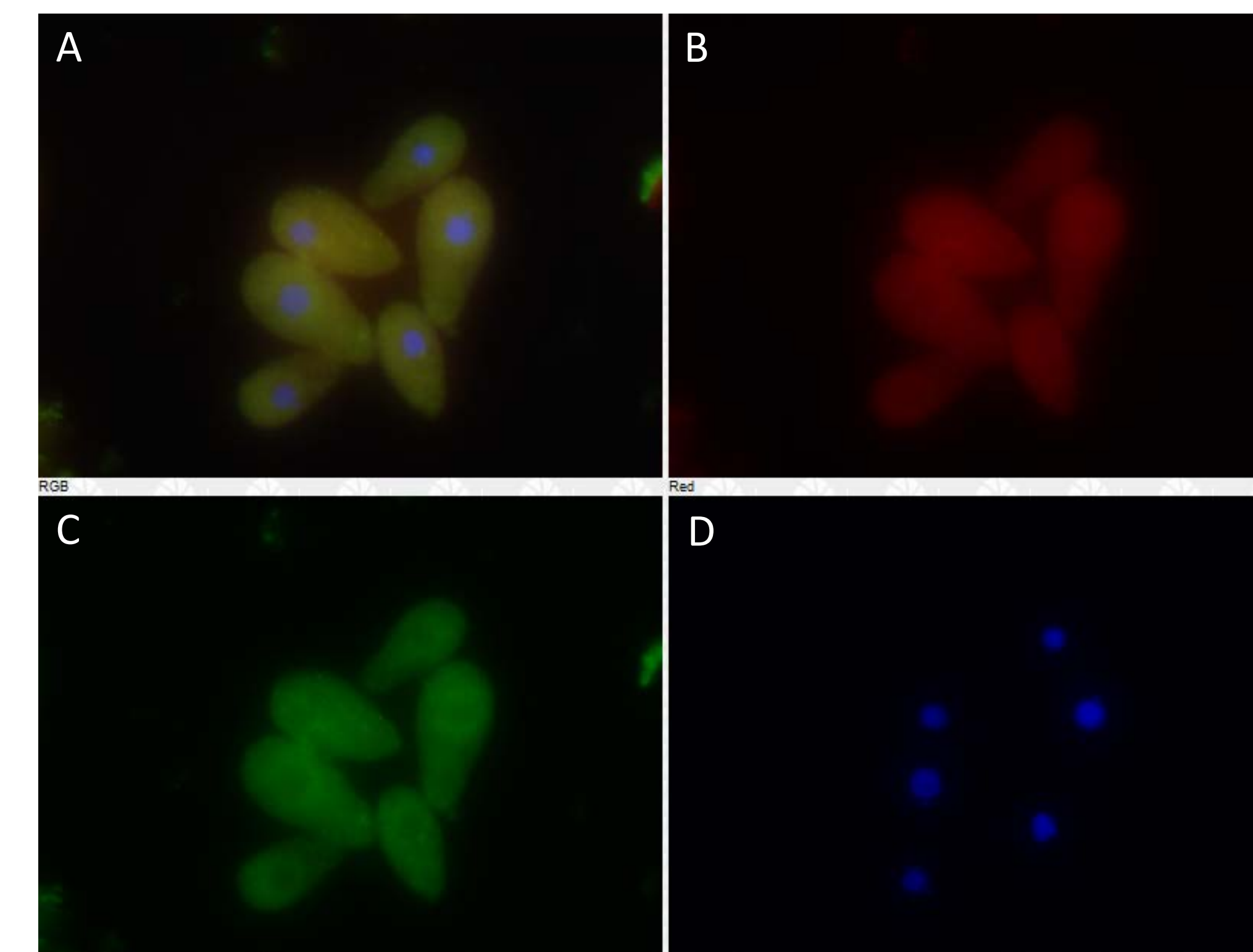


Figure 1. Immunofluorescence shows netrin-1 staining in the cytosol of *Tetrahymena thermophila*. Netrin-1 staining is shown in red (B), tubulin is shown in green (C), and nuclei are shown in blue (D). Panel A is a merge of all three images.

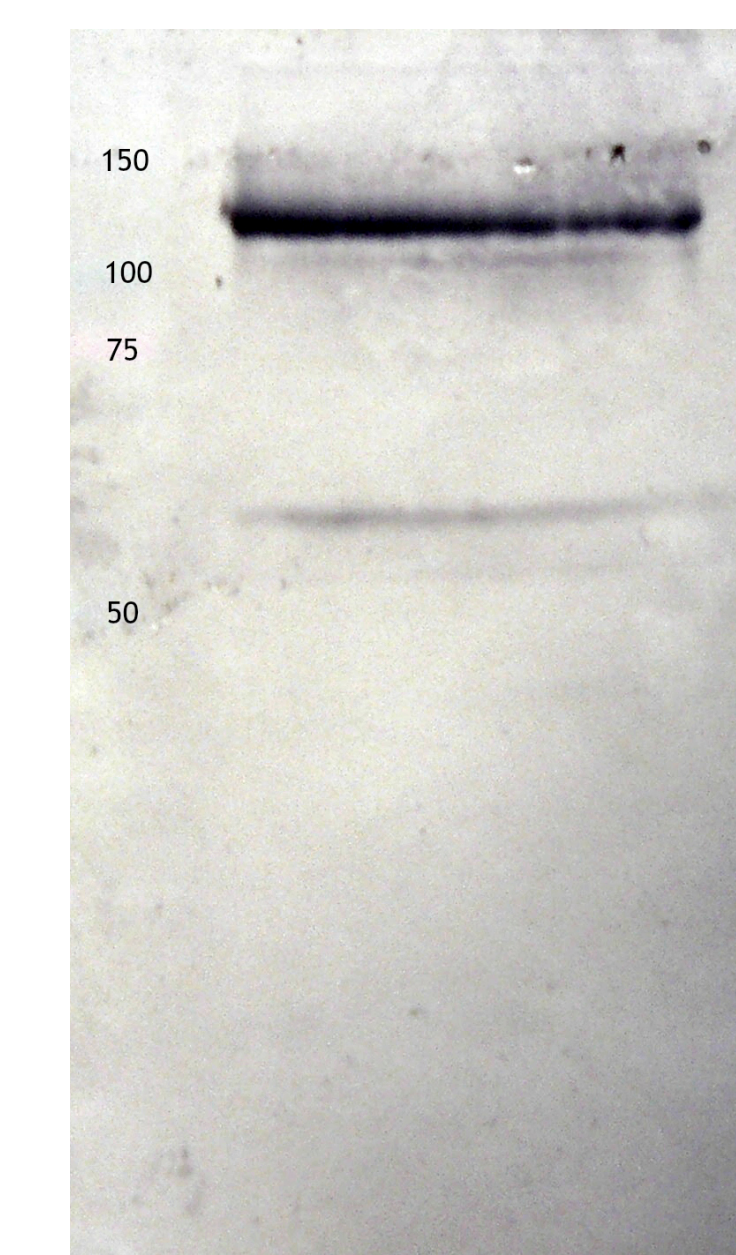


Figure 2. Western Blotting shows evidence of netrin-1 in whole cell extracts obtained from *Tetrahymena thermophila*. Numbers represent molecular weight standards, in kD. The lower molecular weight protein ran at approximately 60 kD and the larger molecular weight protein ran near 120 kD, implying that the larger protein could be a dimer of the smaller protein.

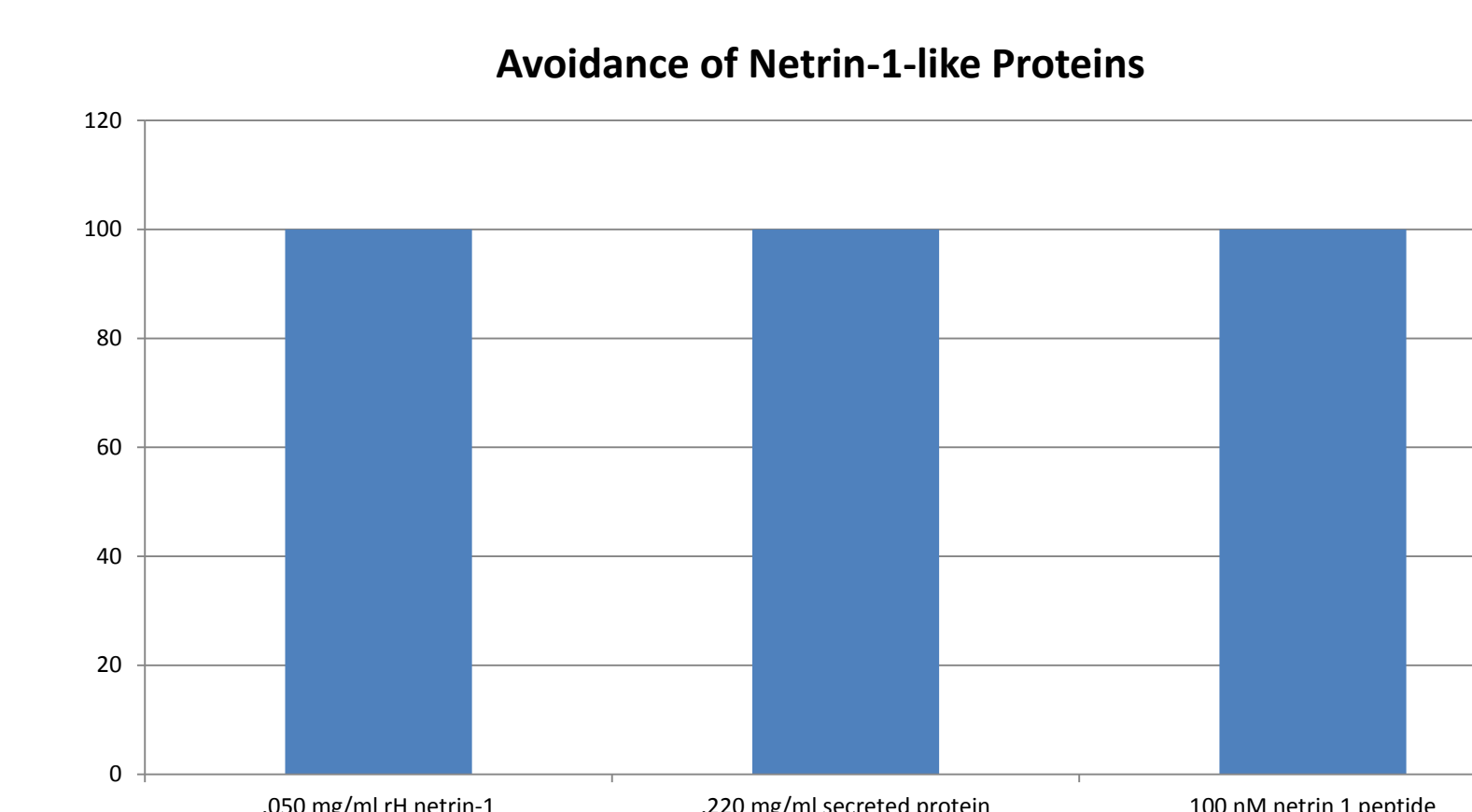


Figure 3. Secreted protein, recombinant netrin-1, and netrin-1 peptide are all chemorepellents in *Tetrahymena thermophila*.

Conclusion

- ELISA shows that reactivity to netrin-1 is present in whole cell extract obtained from *T. thermophila*. Secreted protein had a higher ratio of netrin-1 to total protein when compared with whole cell extract. **This suggests that a netrin-1-like protein is secreted by *T. thermophila*.**
- Netrin-1 immunolocalized to the cytosol of *T. thermophila*. **This suggests that a netrin-1-like protein may play an intracellular role in this organism.**
- Western blotting showed two bands with molecular weights of approximately 60 and 120 kD. **This confirms that a netrin-like protein is present in whole cell extract obtained from *T. thermophila*.**
- **Secreted *Tetrahymena* proteins, recombinant netrin-1, and netrin-1 peptide are all chemorepellents in *T. thermophila*.** Netrin-1 often serves as a chemorepellent during axon guidance in humans, raising the possibility of a shared signaling mechanism.

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Contact Information

Heather G. Kuruville, Professor of Biology
Cedarville University
heatherkuruville@cedarville.edu