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Original Communication

Characterization of a netrin-1-like protein secreted by *Tetrahymena thermophila*

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ABSTRACT

Netrins are a family of pleiotropic signaling proteins, expressed throughout the animal kingdom, that have guidance functions in the development of the nervous system and other branched tissues. Netrins often serve a chemotactic role, acting as chemoattractants or chemorepellents depending upon the type of receptors expressed within the tissue. Chemorepellent transduction usually involves the UNC-5 family of receptors along with the tyrosine kinase, src-1. The best-characterized netrin in the family, netrin-1, has previously been shown to be a chemorepellent in the ciliated protozoan *Tetrahymena thermophila*, and the tyrosine kinase inhibitor genistein, blocked netrin-1 signaling in this organism. *T. thermophila* secrete a protein that is immunologically similar to netrin-1, suggesting that this netrin-1-like protein may play a role in intercellular communication. In this study, we find that the netrin-1-like protein of *Tetrahymena* is a basic protein, approximately 52 kD, which is found in whole cell extract but enriched in secreted protein. In addition, our data indicate that *T. thermophila* have proteins that are immunologically similar to src-1 and UNC-5, suggesting that parts of the netrin signaling pathway conserved throughout the animal kingdom may also be present in Kingdom Protista. Further characterization will be necessary to learn more about these signaling proteins and their physiological role in this organism.

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KEYWORDS: netrin, *Tetrahymena thermophila*, tyrosine kinase, src, UNC-5, UNC-6.

INTRODUCTION

The netrins are a family of signaling proteins with an incredibly diverse array of physiological functions. The name “netrin” comes from the Sanskrit word “netr”, meaning “one who guides” [1], and refers to the early characterization of netrins as axonal guidance molecules [2, 3]. The best-characterized netrin in this family, netrin-1, is expressed throughout the animal kingdom, and is involved in the development of branched tissues, including the lungs [4], vascular system [5] and mammary glands [6]. Netrin-1 has been implicated both positively and negatively in the development of angiogenesis and metastasis in a number of tumor types [5, 7-9], and also acts as a survival factor in some tissue types [10].

Some developmental effects of netrins, such as axonal guidance, are mediated by chemotactic events. Early characterization of netrin-1 showed that it could act as a chemorepellent or a chemoattractant, depending upon concentration and cell type [2, 11]. This was later discovered to be due to the expression of different receptors [4, 12, 13]. More recently, netrin chemotaxis has been found to play important roles in immunological function; netrin-1 augments chemokinesis in CD4+ cells [14], and can modulate the inflammatory response [15].

Chemorepellent signaling in netrin-1 is mediated by receptors belonging to the UNC-5 family, which either form homodimers, or heterodimerize with the DCC receptor [16]. In *Caenorhabditis elegans*, as well as some vertebrate systems, the UNC-5 signal is mediated through the tyrosine kinase src-1 [16, 17].

Tetrahymena thermophila are free-living, unicellular eukaryotes. Since they are motile cells, they have been used as models for chemotransduction for decades [18-22]. When exposed to a repellent, cells will reverse their cilia, resulting in a jerky, irregular swimming pattern [22]. *Tetrahymena* avoidance behavior can be viewed and quantitated under a simple dissection microscope.

We have previously shown that netrin-1 is a chemorepellent in *Tetrahymena thermophila*, and that *Tetrahymena* appear to secrete a netrin-1 -like protein, as determined by enzyme-linked immunosorbent assay (ELISA) [23]. In this study, we have further characterized this netrin-1-like protein and its putative signaling pathway in order to better understand its physiological roles in this organism.

MATERIALS AND METHODS

Cell cultures

Tetrahymena thermophila, strain B2086.2, were obtained from the Tetrahymena Stock Center at Cornell University https://tetrahymena.vet.cornell.edu/.Cells were grown at 20 °C in the medium of Dentler [24] without shaking or addition of antibiotics. One-day-old cell cultures were used for all behavioral and pharmacological assays.

Chemicals and solutions

Netrin-1 peptide and anti-netrin-1 antibody were obtained from Abcore LLC, Ramona, CA. Recombinant human netrin-1 was obtained from R&D Systems, Minneapolis, MN. ER Tracker™ was obtained from ThermoFisher Scientific, Waltham, MA. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Phosphate buffered saline (PBS) was made by dilution of a 10X stock with distilled water. Behavioral buffer consisted of 10 mM Trizma Base, 0.5 mM MOPS, 50 µM $CaCl_2$; pH was adjusted to 7.0 with hydrochloric acid.

Behavioral assays

Behavioral assays were carried out as previously described [22]. *Tetrahymena thermophila* were washed 3 times in behavioral buffer and then 300 µl of cell suspension was transferred to the first well of a microtiter plate. Cells were then transferred individually using a micropipette into the second well of the microtiter plate, already containing 300 µl of buffer as a control. Cells were then individually transferred to a third well containing 300 µl of the protein being tested for chemorepellent activity. Behavior of the individual cells was observed for the first few seconds after they were added to the well, and the percentage of cells exhibiting avoidance behavior was noted.

Western blotting

Cell extracts were prepared for Western blotting as follows: 2-day-old cell cultures were harvested and washed twice in behavioral buffer. Cell pellets were extracted on ice for 60 minutes in 0.5% Triton X-100 in the presence of protease inhibitor cocktail. After extraction, mixture was centrifuged at 4 °C in a microcentrifuge for 15 minutes at 12,500 rpm. Pellets were discarded; supernatants were run on a 10% SDS-PAGE. Gels were transferred to a nitrocellulose membrane and blocked overnight in PBS-Tween containing 1% bovine serum albumin (BSA). Western blots were performed using a 1:1000 dilution of goat anti-netrin-1 IgG as the primary antibody and a 1:5000 dilution of rabbit-anti-goat IgG, alkaline phosphatase conjugate, as the secondary antibody. Nitro blue tetrazolium substrate was used to show alkaline phosphatase activity.

Ion exchange chromatography

CM-Sephadex C-50 was equilibrated to pH 7.0 using behavioral buffer. One ml whole cell extract was applied to a 25 ml column of CM-Sephadex C-50. The column was then washed with 2 volumes (50 ml) behavioral buffer at pH 7.0. Finally, PBS, pH 10, was added to the column in order to elute the proteins of interest. Fractions were collected in increments of 6 ml, precipitated using chloroform/ methanol, and used for Western blotting.

Immunofluorescence

Cells from 2-day-old cultures were washed twice in behavioral buffer, reconstituted in 3.7%

formaldehyde in behavioral buffer, and fixed for 15 min at room temperature. After fixation, cells were rinsed three times in PBS before being incubated in blocking buffer (PBS containing 0.3% Triton X-100 and 3% BSA) for 60 minutes. After rinsing with PBS, cells were incubated overnight at room temperature in primary antibody at a dilution of 1:100 in the presence of antibody dilution buffer (PBS containing 0.3% Triton X-100 and 1% BSA). After rinsing three times in PBS, cells were incubated with a 1:100 dilution of fluorochrome-conjugated secondary antibody for 1-2 hours at room temperature in the dark, in the presence of antibody dilution buffer. Cells were then rinsed three times in PBS. 10 µl of cell suspension was then applied to a slide and mixed with 10 µl 4',6-diamidino-2-phenylindole (DAPI). The cell suspension was then covered with a coverslip and observed under a Nikon fluorescence microscope at 400X. Photographs were taken with a QI Click camera using NIS Elements Software, version 4.40. Fluorescence was quantitated using NIS Analysis software, version 4.40.

RESULTS

Our previous work with *Tetrahymena thermophila* showed that netrin-1-like peptides act as chemorepellents in this organism, and that *Tetrahymena* secrete a netrin-1-like protein. In light of these findings, we hypothesized that secreted proteins from *Tetrahymena* would also act as chemorepellents in this organism. We also hypothesized that whole, recombinant netrin-1 would be a chemorepellent. Using behavioral assays, we found that *Tetrahymena* exposed to 0.050 mg/ml recombinant human netrin-1, 0.220 mg/ml secreted protein from *Tetrahymena*, and 100 nM netrin-1 peptide all showed 100% avoidance in our behavioral assay (Figure 1).

Since a netrin-1-like protein is secreted from *Tetrahymena*, we hypothesized that this protein

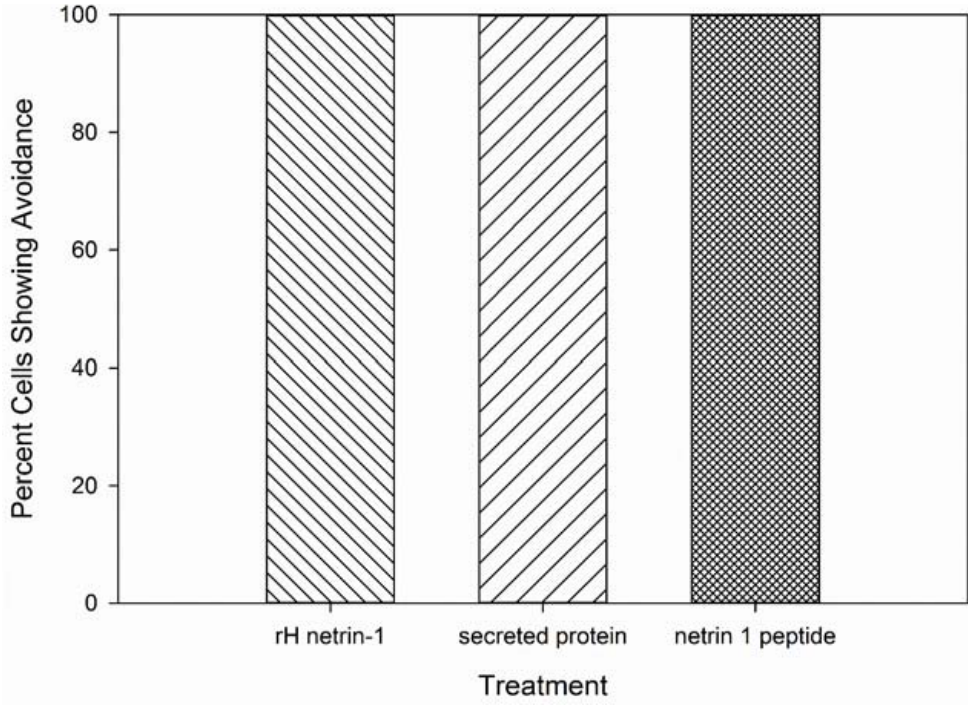

Figure 1. Recombinant human netrin 1, *Tetrahymena*-secreted protein, and netrin-1 peptide are all effective chemorepellents in *Tetrahymena thermophila*. Behavioral avoidance is caused by 0.050 mg/ml recombinant human netrin-1, 0.220 mg/ml secreted protein from *Tetrahymena*, and 100 nM netrin-1 peptide. Each trial represents 10 cells, which were individually scored as being either positive or negative for avoidance. Each bar represents the mean $\pm$ SD of ten cells.

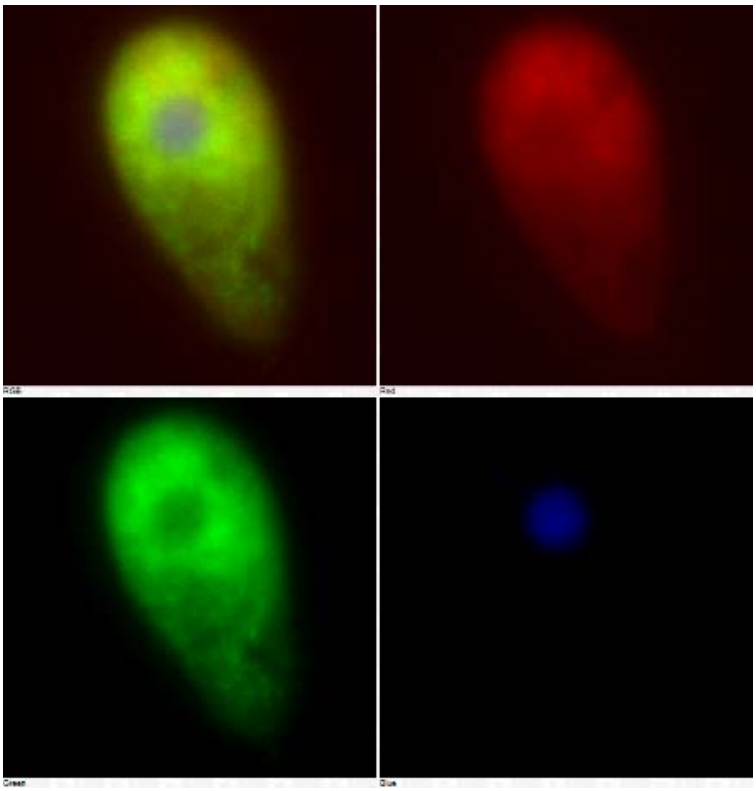

Figure 2. Netrin-1 colocalizes with ER Tracker, as would be expected for a secreted protein**.** Immunolocalization was done with a polyclonal anti-netrin-1 antibody conjugated to AlexaFluor 488 (green; bottom left). Cells were counterstained with ER Tracker (red; top right) and DAPI (blue; bottom right). Significant colocalization is seen between the anti-netrin-1 antibody and ER tracker (RGB; top left), consistent with a protein that is synthesized in the ER and trafficked through the secretory pathway.

might be found in the endoplasmic reticulum and Golgi apparati, since its synthesis would likely follow the secretory pathway. Immunolocalization with an anti-netrin-1 antibody showed strong colocalization with ER Tracker™ (Figure 2), as was expected for a secreted protein.

In order to determine the molecular weight of the netrin-1-like protein in *Tetrahymena*, we prepared extracts from whole *Tetrahymena* cells as well as secreted protein extracts. Western blotting of these extracts using an anti-netrin-1 antibody revealed several bands. A band which ran at approximately 52 kD was enriched in secreted protein when compared with whole cell extract (Figure 3) even though 5 µg total protein was loaded in each experimental lane. Our positive control, recombinant human netrin-1, ran at 75 kD.

Using the Uniprot database (www.uniprot.org), we determined that human netrin-1 is a basic protein, carrying a net positive charge at pH 7. Based on these findings, we hypothesized that the netrin-1-like protein of *Tetrahymena* might also carry a positive charge. Using ion-exchange chromatography, we partially purified secreted proteins from *Tetrahymena* to remove acidic proteins from the extract. When we ran these proteins on a Western blot, the predominant band ran at approximately 52 kD (Figure 4).

In the animal kingdom, transduction of a chemorepellent signaling *via* netrin-1 often involves the UNC-5 family of receptors, which are associated with the tyrosine kinase, src-1. Since our previous work showed that inhibiting tyrosine kinases prevents *Tetrahymena* from responding to netrin-1 peptide, we hypothesized that *Tetrahymena* might possess homologous signaling proteins. We retrieved the amino acid sequence for src-1 in *C. elegans* (G5EE6) from http://www.uniprot.org/ and used

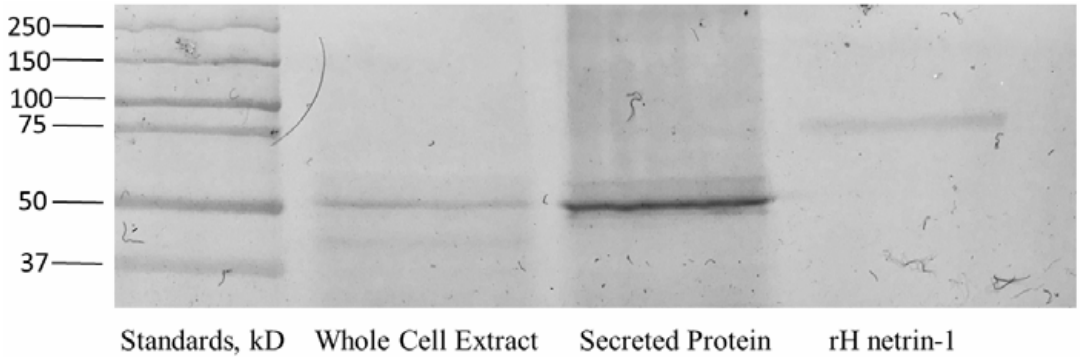

Figure 3. Netrin-1-like protein is found in both whole cell extract and secreted protein obtained from *Tetrahymena.* Proteins in each lane were detected using an anti-netrin-1 antibody. When compared to whole cell extract, the secreted protein fraction is enriched in a protein that runs at approximately 52 kD. 5 µg of total protein was added to each experimental well. The positive control, recombinant human netrin-1, ran at approximately 75 kD.

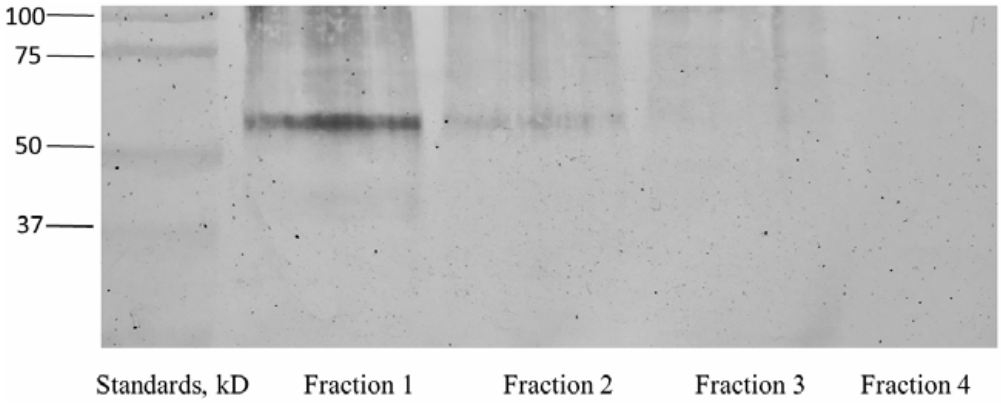

Figure 4. Netrin-1-like protein found in *Tetrahymena* is a basic protein which binds to a CM-Sephadex ion exchange resin. Proteins from whole cell extract were allowed to bind to the ion exchange resin at a pH of 7 and eluted from the resin at a pH of 10. Fractions were precipitated using chloroform/methanol and then reconstituted before being run on a Western blot using anti-netrin-1 antibody.

the blastp program to search the *T. thermophila* amino acid database at http://www.ciliate.org/blast/blast_link.cgi. This search yielded 102 hits, including four tyrosine kinase domain proteins: TTHERM_00140760, TTHERM_00578620, TTHERM_00426220, and TTHERM_00195930. Using the same databases, we retrieved the amino acid sequence for UNC-5 in *C. elegans* (Q26261), which we used to search the *T. thermophila* amino acid database. This search produced 4 hits, including MCM4, an ABC transporter family protein, a hypothetical protein TTHERM_00055940, and a serine/threonine kinase domain protein TTHERM_00440630.

Since our database search confirmed that *Tetrahymena* may express proteins similar to src-1 and UNC-5, we used ELISA and immunofluorescence to determine if *Tetrahymena* express proteins that are immunologically similar to src-1 and UNC-5. As seen in Table 1, ELISA of whole cell extract indicates that *Tetrahymena thermophila* express proteins that are immunologically similar to src-1 and UNC-5. Fluorescence of whole cell extract with an anti-src antibody was 7.08 times that of the BSA control. A two-tailed T-test showed this was significantly higher than the control, with a P value of 0.0002. Fluorescence of whole cell extract with an

Table 1. ELISA indicates that proteins similar to src and UNC-5C are present in *Tetrahymena thermophila.* Whole cell extract from 2-day-old *Tetrahymena* cultures were used for ELISA. Antibody binding to whole cell extract was compared to antibody binding to 1% BSA, which was used as a control for nonspecific binding. A two-tailed T-test showed that fluorescence obtained with the anti-src-5 antibody was significantly higher than that of the BSA control (P = 0.01) and that fluorescence obtained with the anti-UNC-5C antibody was significantly higher than that of the BSA control (P = 0.002).

	Mean fluorescence	**Relative fluorescence**
Anti-src antibody, BSA control	$7.70e^3 \pm 1.10e^2$	--
Anti-src antibody, whole cell extract	$5.45e^4 \pm 1.15e^3$	7.08
Anti-UNC-5C antibody, BSA control	$9.59e^3 \pm 3.84e^2$	--
Anti-UNC-5C antibody, whole cell extract	$2.38e^4 \pm 2.72e^3$	2.48

anti-UNC-5C antibody was 2.48 times that of the BSA control. A two-tailed T-test showed this was significantly higher than the control, with a P value of 0.01.

We used immunofluorescence as an alternative method of determining whether *Tetrahymena* express proteins that are immunologically similar to src and UNC-5. Fluorescence intensity of cells stained with anti-src (Figure 5A) and anti-UNC-5C (Figure 5B) was compared with controls that were stained with secondary antibody alone (Figure 5C). The mean fluorescence of cells stained with anti-src and UNC-5C antibodies was significantly higher than that of the secondary antibody controls, with P values of < 0.0003 for both sample groups. Diffuse staining was seen with both antibodies, consistent with cytosolic or plasma membrane staining, along with punctate staining consistent with the endomembrane system. We did not see staining of the cilia with either the anti-src or anti-UNC-5C antibodies.

DISCUSSION

Since secreted proteins from *Tetrahymena* have previously been shown to contain a netrin-1-like protein [23], and since netrin-1 peptide is a chemorepellent in *Tetrahymena* [23], we hypothesized that full-length recombinant netrin-1, as well as secreted proteins from *Tetrahymena* containing a netrin-1-like peptide, would act as chemorepellents in *Tetrahymena.* Our behavioral assay showed that all three compounds cause 100% avoidance in *Tetrahymena* at the concentrations tested (Figure 1). Using the netrin homologue, UNC-5 from *C. elegans* (P34710), we used the blastp program to search the *T. thermophila* amino acid database at http://www.ciliate.org/blast/blast_link.cgi. This search yielded 237 hits, four of which had E values of 0.0003 or less. The two top matches were hypothetical protein TTHERM_000323149 and hypothetical protein TTHERM_000629740. It is possible that one of these proteins, or a similar protein, corresponds to the netrin-1-like protein of *Tetrahymena.*

Because of our previous work showing that a netrin-1-like protein was secreted from *Tetrahymena* [23], we hypothesized that the netrin-1-like protein of *Tetrahymena* would be found within the endoplasmic reticulum, as well as in secretory vesicles throughout the cell. Using immunolocalization, we found the netrin-like protein colocalizing with ER Tracker™, as we would expect for a protein that uses the secretory pathway (Figure 2). In addition, our Western blot (Figure 3) shows that a band of approximately 52 kD is enriched in the secreted protein extract when compared with whole cell extract. Like vertebrate netrin, the netrin-1-like protein of *Tetrahymena* appears to be basic protein, since it can be purified using cation exchange on a CM-Sepharose column and then running the proteins on a Western blot (Figure 4). Knowing that the

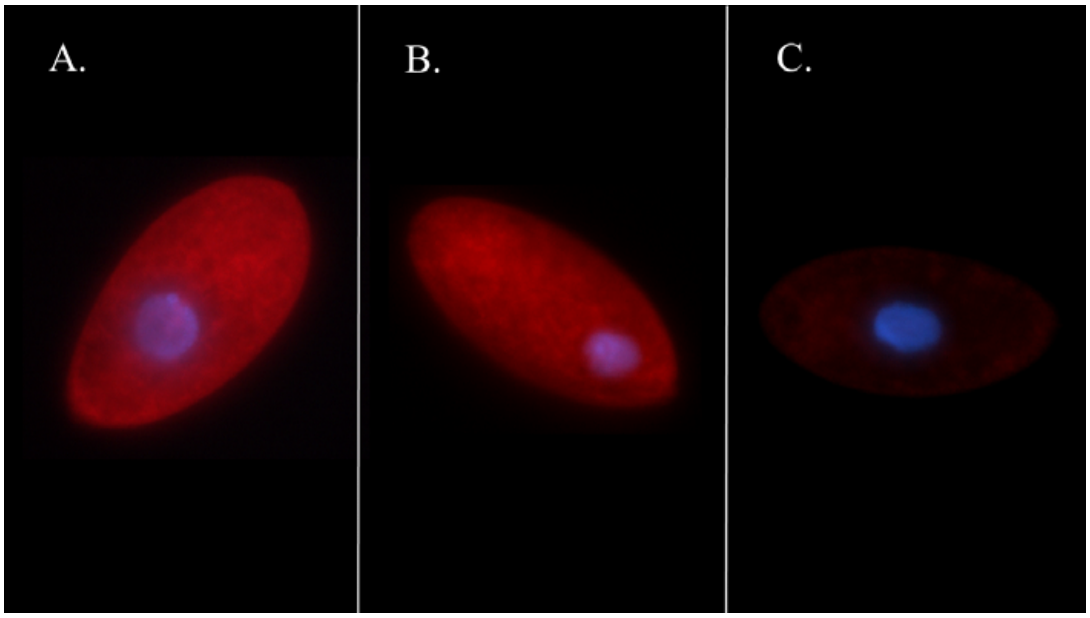

Figure 5. Immunolocalization using anti-src and anti-UNC-5C antibodies in *Tetrahymena thermophila*. Fluorescence intensity of cells stained with (A) anti-src and (B) anti-UNC-5C was compared with (C) controls that were stained with secondary antibody alone. The mean fluorescence of cells stained for src and UNC-5C was significantly brighter than that of the secondary antibody controls in a two-tailed T test, with P values of < 0.0003 for both sample groups.

molecular weight of the protein is approximately 52 kD, and that the net charge at pH 7 is positive, we hope to sort through the large number of hypothetical candidate proteins yielded by our database search to determine which might be the netrin-1-like protein of *Tetrahymena.*

Netrin-1 binds to a number of receptors in vertebrates, and interacts with UNC-5 in *C. elegans* [12, 17]. When signaling as a chemorepellent during axonal guidance, the UNC-5 family receptors are often used along with the tyrosine kinase, src-1 [17, 25]. In addition, in *Tetrahymena*, the chemorepellent activity of netrin-1 peptide is blocked by the addition of the tyrosine kinase inhibitor genistein to the buffer, suggesting that tyrosine kinases are required for netrin-1 signaling [23]. Our ELISA (Table 1) and immunofluorescence data (Figure 5) show that *Tetrahymena* express proteins that are immunologically similar to src and UNC-5. This finding is consistent with the existence of a signaling pathway in *Tetrahymena* that is analogous to the UNC-5/UNC-6 pathway of *C. elegans* and the netrin-1/unc-5 pathway in vertebrates. Our database searches suggest a number of proteins that could be homologs of src-1 and UNC-5. Sequences derived from these candidate proteins may be useful in gene knockout studies, to determine if knocking out a UNC-5 or src-1 homolog in *Tetrahymena* would generate mutants that were unresponsive to netrin-1.

Since *Tetrahymena* are unicellular organisms, the physiological role of a netrin pathway will necessarily be quite different than it would be in a multicellular organism. However, netrin's function as a chemorepellent is common to both vertebrates [11] and *Tetrahymena* [23]. This guidance role of netrins allows for the development of branched tissues in animal systems [4-6]. In *Tetrahymena*, the production and secretion of a netrin-like protein may serve a role in allowing cells to repel one another when the local concentration of cells becomes too high for the available resources. In their natural environment, *Tetrahymena* could use chemorepellent signals from other cells, including netrin-like proteins, along with chemoattractant signals from food sources, to guide them to a location where they have a better chance of survival.

Further research will allow us to better characterize the netrin-1-like protein of *Tetrahymena*, along with its signaling pathway. It may be that netrins are a common guidance molecule throughout living systems, found in other members of Kingdom Protista along with Kingdom Animalia. Perhaps our study of Protista will help divulge a previously undisclosed secret of netrin signaling in the animal world.

CONCLUSION

Tetrahymena thermophila secrete a basic protein, approximately 52 kD in molecular weight, that is immunologically similar to vertebrate netrin-1. *Tetrahymena* also express proteins that are immunologically similar to src and UNC-5C. Our data are consistent with the existence of a signaling pathway in *Tetrahymena* that is analogous to the UNC-6/netrin signaling pathway in *C. elegans*, and the netrin-1 signaling pathway in vertebrates.

CONFLICT OF INTEREST STATEMENT

The authors whose names are listed on the title page certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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