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Netrin-3 Avoidance and Mitotic Inhibition in *Tetrahymena thermophila* Involves Intracellular Calcium and Serine/Threonine Kinase Activity

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Abstract

Netrins are a family of signaling proteins ubiquitously expressed throughout the animal kingdom. While netrin-1 has been well characterized, other netrins, such as netrin-3, remain less well understood. In our current study, we characterize the behavior of two netrin-3 peptides, one derived from the N-terminal and one derived from the C-terminal of netrin-3. Both peptides cause avoidance behavior and mitotic inhibition in *Tetrahymena thermophila* at concentrations as low as 0.5 μg/ml. These effects can be reversed by addition of the calcium chelator, EGTA; the intracellular calcium chelator, BAPTA-AM, or the serine/threonine kinase inhibitor, apigenin. The broad spectrum tyrosine kinase inhibitor, genistein, has no effect on netrin-3 signaling, indicating that netrin-3 signaling in this organism uses a different pathway than the previously described netrin-1 pathway. Further studies will allow us to better describe the netrin-3 signaling pathway in this organism.

Materials and Methods

Behavioral assays were carried out as described in Mace *et al*., 2000. Pharmacological inhibition assays were performed similarly to the behavioral assays described in Keedy *et al*., 2003. Immunofluorescence was carried out using a modified protocol obtained from cellsignal.com. 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 a 1:100 dilution of secondary antibody for 1–2 hours at room temperature in the dark. Cells were then rinsed three times in PBS. 5 μl of cell suspension was then applied to a slide and mixed with 5 μl of DAPI. Cell suspension was then observed under a fluorescence microscope at 400X.

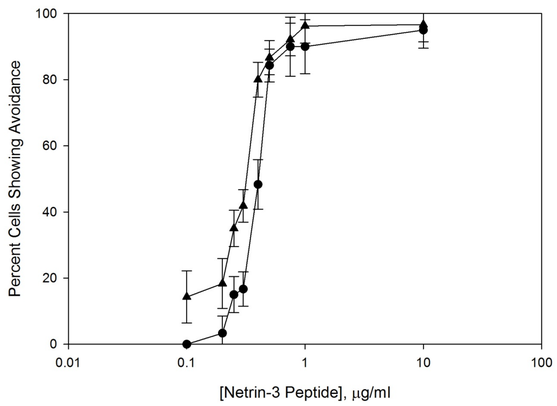
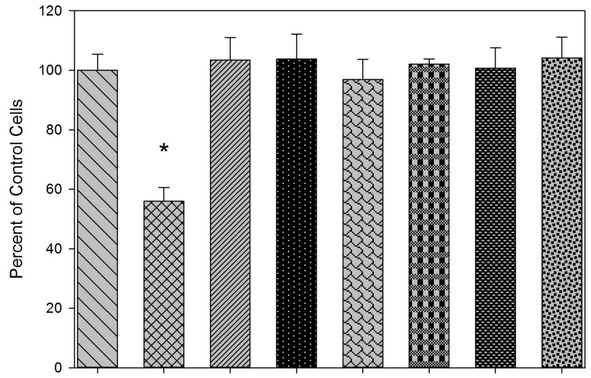


Figure 1. Both Netrin-3 C terminal (triangles) and Netrin-3 N-terminal (circles) are chemorepellents in *Tetrahymena thermophila* in the micromolar range. Each point represents 6 sets of data. Each set represents the mean ± SD of ten cells, which were individually scored for avoidance. The potencies of the two peptides were similar., and both peptides appear to use the same signaling pathway (data not shown). Since we had more N3C peptide, this peptide was used in our remaining pharmacological and mitosis assays.

Results

Table 1. Comparison of Netrin-1 and Netrin-3 Signaling in *Tetrahymena thermophila*. Pharmacological inhibitors were used to block chemorepellent signaling. Netrin-1 signaling in *Tetrahymena* is similar to signaling through the DCC receptor in vertebrates, while netrin-3 signaling is similar to signaling through the UNC-5 receptor in vertebrates.

Pharmacological Inhibitor	Mechanism of Action	Inhibits N-1 Avoidance	Inhibits N3 Avoidance
Pertussis Toxin	Inhibits Gi/o proteins	No	No
GDP-B-S	Inhibits G proteins	No	No
EGTA	Chelates extracellular calcium	No	Yes
BAPTA-AM	Chelates intracellular calcium	No	Yes
Genistein	Tyrosine kinase inhibitor	Yes	No
Hypericin	Tyrosine kinase inhibitor	Yes	No
Vanadate	Tyrosine Phosphatase Inhibitor	No	Yes
Apigenin	Broad Spectrum kinase inhibitor	Yes	Yes



Left to right: Control, Netrin-3 alone, EGTA alone, EGTA + N-3, BAPTA alone, BAPTA + N-3, Apigenin alone, Apigenin + N-3

Figure 2. Mitosis is inhibited by netrin-3 peptide, and rescued by pharmacological agents that inhibit netrin-3 avoidance. This suggests that netrin-3 uses the same signaling pathways to inhibit mitosis as it does to cause avoidance behavior. Inhibition of mitosis was significantly different when compared with controls using a two-tailed T test, with a P-value of 4.13×10^{-6}.

Table 1. Comparison of Netrin-1 and Netrin-3 Signaling in *Tetrahymena thermophila*. Pharmacological inhibitors were used to block chemorepellent signaling.

	Control Cells	Netrin-3 Treated Cells
Mean Nuclear Radius, nM	12.89	14.29
Median Nuclear Radius, nM	12.68	14.54
T-test Result	------	0.00028

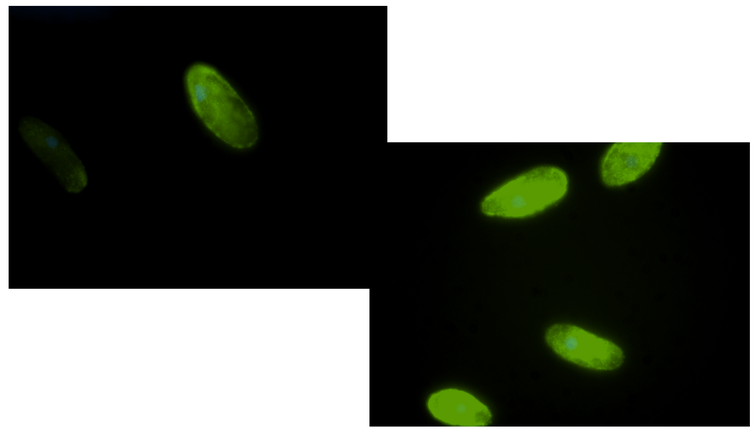


Figure 3. Netrin-3 treatment causes an increase in phosphoserine levels in *Tetrahymena thermophila*. Netrin-3 treated cells (right) show an increased level of phosphoserine levels when compared to control cells (left). Immunofluorescence was conducted using an anti-phosphoserine antibody. Green staining indicates the presence of phosphoserine; blue staining indicates the presence of DAPI.

Conclusions

- Netrin-3 peptide is a chemorepellent in *Tetrahymena thermophila* in the micromolar range.
- Netrin-3 signaling in *Tetrahymena thermophila* is calcium dependent and appears to involve serine phosphorylation. G-proteins and tyrosine kinases do not appear to play a role in avoidance behavior.
- Netrin-3 inhibits mitosis in *Tetrahymena thermophila.* This inhibition is reversed in the presence of the broad spectrum kinase inhibitor, apigenin, and in the presence of calcium chelators.
- There is a significant increase in mean nuclear size in *Tetrahymena* grown in the presence of netrin-3. This indicates that a greater proportion of cells are in the G_2 phase of growth when compared to control cells.

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References

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2. Keedy, M. D., Yorgey, N. K., Hilty, J. S., Price, A. R., Hassenzahl, D. L., & Kuruvilla, H. G. (2003). Pharmacological evidence suggests that the lysozyme/PACAP receptor of Tetrahymena thermophila is a polycation receptor. *Acta Protozoologica*, *42*(1), 11.