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Netrin-1 Signaling in *Tetrahymena thermophila*: The Tyrosine Kinase Controversy Continues

Matthew S. Merical
Cedarville University, mmerical@cedarville.edu

Heather G. Kuruvilla
Cedarville University, heatherkuruvilla@cedarville.edu

Netrin-1 Signaling in *Tetrahymena thermophila*: The Tyrosine Kinase Controversy Continues

Matthew S. Merical and Heather G. Kuruvilla
Department of Science and Mathematics, Cedarville University, Cedarville, OH 45314 USA

Abstract

Netrin-1 is a pleiotropic signaling molecule first discovered for its role in neuronal development, where it is largely responsible for axonal guidance. When signaling through the DCC receptor, netrin-1 serves as a chemoattractant; however, signaling through the UNC5 receptor results in chemorepulsive activity (Ko et al., 2012). In the free-living ciliate, *Tetrahymena thermophila,* netrin-1 and netrin-1 peptide act as chemorepellents at micromolar to nanomolar concentrations, causing cells to exhibit avoidance behavior. While many pharmacological inhibitors that we tested had no effect on avoidance behavior, the tyrosine kinase inhibitor, genistein (IC_{50} ~ 50 μg/ml), inhibited avoidance behavior in this organism. However, when using the monoclonal anti-phosphotyrosine antibody PT-66 to probe for phosphotyrosine in control and netrin-1 exposed cells, both control and netrin-1 exposed cells showed a low level of immunostaining, indicating that tyrosine phosphorylation was not required for netrin signaling. The localization of the staining was also similar in both groups. Genomic studies of *Tetrahymena thermophila* (Eisen et al., 2006) indicate that no tyrosine kinases are found in this organism. Previous biochemical studies (Christensen et al., 2003; Bartholomew et al., 2008) have suggested that tyrosine kinases are required for signaling in this organism. Our current data imply that the tyrosine kinase inhibitors we used may be binding to an alternative site in *Tetrahymena.* Additional studies will be necessary in order to determine the mechanism by which tyrosine kinase inhibitors are blocking netrin-1 avoidance in this organism.

Introduction

The netrin family of proteins are highly conserved pleiotropic signaling molecules which belong to the laminin superfamily. Netrins are present in all bilaterally symmetrical animals studied to date, and receptors for netrins have been found in all vertebrates studied thus far. The most well characterized netrin, netrin-1 was first noted for its effects on axonal guidance. When signaling through DCC, DSCAM, or neogenin, netrin-1 may act as a chemoattractant; however, when signaling through UNC-5 or a heterodimer of DCC and UNC-5, netrin acts as a chemorepellent.

Much effort has gone into understanding the various signaling pathways through which netrin-1 works. Signaling mechanisms are complex, given the number of receptors netrin-1 binds to, as well as the multifaceted physiological roles attributed to netrin-1. Chemorepellent signaling is thought to involve the tyrosine kinase, src-1, and the tyrosine phosphatase, shp-2.

Tetrahymena thermophila are free-living, eukaryotic ciliates often used as a model system for chemorepellent signaling. When exposed to a chemorepellent*, Tetrahymena* reverse their cilia, causing them to exhibit avoidance behavior which is characterized by swimming in circles or jerky, back and forth swimming. This behavior is easily observed and characterized under a light microscope. *Tetrahymena* avoid a number of polycationic proteins and peptides, including lysozyme , PACAP-38 , and nociceptin.

Since netrin-1 acts as a chemorepellent in some vertebrate cell types, and since netrin-1 peptide is polycationic under our assay conditions, we believe that netrin-1 peptide will act as a chemorepellent in *Tetrahymena thermophila*.

Materials & Methods

Behavioral Assays
Behavioral assays were carried out as previously described (Mace *et al*., 2000; Robinette *et al*., 2008). Adaptation and cross-adaptation assays were carried out as previously described (Keedy *et al.*, 2003). Pharmacological inhibition assays were performed similarly to the behavioral assays described above (Keedy *et al*., 2003).

Immunofluorescence
T. thermophila were washed 3 times in behavioral buffer and then fixed in 3.7% formaldehyde, diluted in behavioral buffer, for 15 minutes. Cells were then washed 3 times in PBS and incubated in blocking buffer overnight. Cells were then washed in PBS and incubated in a 1:100 dilution of either anti-netrin-1 or anti-tubulin antibody in the presence of antibody dilution buffer, for 2 hours with constant shaking. Cells were once again washed in PBS and then incubated with a 1:100 dilution of secondary antibody, for one hour with constant shaking. Cells were then washed in PBS, stained with DAPI, and viewed under a Nikon H550L Microscope using the Nikon Intensilight C-HGFI. Fluorescence images were obtained with a QI Click 74-0083-AO camera using NIS Elements BR 4.13.04 Software.

Results

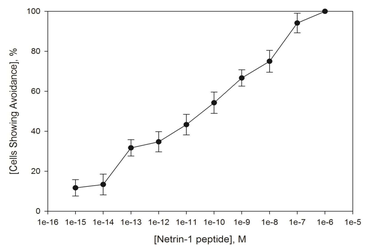

Figure 1. Netrin-1 peptide is a chemorepellent in *Tetrahymena thermophila*. The EC_{100} of this peptide is 1 μM. The EC_{50} of this peptide is approximately 1 nM. "Cells Showing Avoidance " represents the mean of at least 6 trials, and error bars represent the standard deviation. Each trial consisted of 10 cells which were individually observed and scored for avoidance.

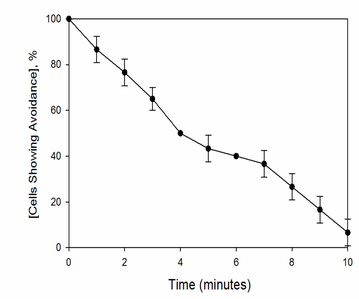

Figure 2. Time course of adaptation to netrin-1 peptide in *Tetrahymena thermophila*. Adaptation studies were done at 1 μM netrin-1 peptide, which is the EC_{100} of this peptide. "Cells Showing Avoidance" represents the mean of at least 6 trials, and error bars represent the standard deviation. Each trial consisted of 10 cells which were individually observed and scored for avoidance.

Table 1. Cells adapted to netrin-1 peptide are not cross-adapted to nociceptin, GTP, or PACAP-38. Percentage avoidance, as listed below, represents the mean ± standard deviation of at least 6 trials. Each trial consisted of 10 cells which were individually observed and scored for avoidance. Adaptation to the same signal (e.g. GTP adapted to GTP, nociceptin adapted to nociceptin, etc.) were run as controls. Each of these controls showed adaptation, showing less than the baseline avoidance of 20% typically seen in behavioral assays.

	GTP	PACAP-38	Nociceptin	Netrin-1 Peptide
GTP	13.3 ± 5.8	95.0 ± 5.0	94.0 ± 5.2	93.0 ± 4.1
PACAP-38	96.6 ± 5.2	12.5 ± 9.6	97.5 ± 4.6	95.0 ± 5.5
Nociceptin	96.6 ± 5.8	100 ± 0.0	9.2 ± 8.2	95.0 ± 5.0
Netrin-1 peptide	90.0 ± 10.0	90.0 ± 0.0	93.3 ± 5.8	6.66 ± 5.8

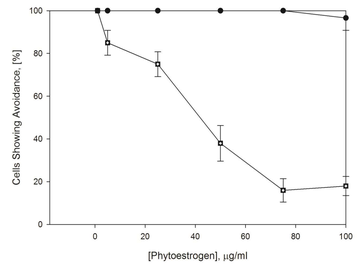

Figure 3. Avoidance of netrin-1 peptide is inhibited by the tyrosine kinase inhibitor, genistein, but not by daidzein, a negative control for genistein activity. The IC_{50} of genistein is approximately 50 μg/ml. Baseline avoidance was achieved at a genistein concentration of 75 μg/ml. Daidzein had no effect on avoidance behavior. Percentages represent the mean ± standard deviation of at least 6 trials. Each trial consisted of 10 cells which were individually observed and scored for avoidance.

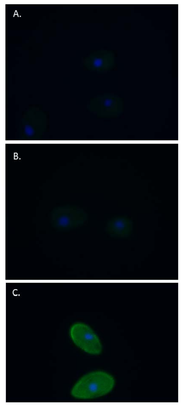

Figure 4. Tyrosine phosphorylation levels are not affected by netrin-1 peptide. Indirect immunofluorescence using PT-66 anti-phosphotyrosine antibody shows no difference in fluorescence intensity between control (A) and netrin-1 exposed cells (B). This indicates that tyrosine phosphorylation is not required for netrin-1 signaling. In constrast, cells stained with an anti-tubulin antibody (C) show a high level of fluorescence intensity.

Conclusions

- Netrin-1 peptide is a chemorepellent in *Tetrahymena thermophila,* causing the cells to exhibit avoidance reactions.
- Cells adapt to netrin-1 peptide over a time course of approximately 10 minutes, similar to the time course seen for other chemorepellents such as PACAP-38 and GTP.
- Cross-adaptation studies indicate that netrin-1 peptide uses a different signaling mechanism than those used by the chemorepellents GTP, PACAP-38, or nociceptin.
- Avoidance of netrin-1 peptide is blocked by addition of the broad spectrum tyrosine kinase inhibitor, genistein, to the behavioral buffer. However, addition of diadzein to the buffer does not affect avoidance.
- Immunostaining using an anti-phosphotyrosine antibody shows no difference in staining intensity between control cells and cells exposed to netrin-1 peptide, giving no evidence of tyrosine kinase activity in this signaling pathway.
- Further experimentation is needed to determine the mechanism of action of genistein in *Tetrahymena thermophila.*

References

1.Ko, S. Y., Dass, C. R., & Nurgali, K. (2012). Netrin-1 in the developing enteric nervous system and colorectal cancer. *Trends in molecular medicine*, *18*(9), 544-554.

2. Eisen, J.A., Coyne, R.S., Wu, M., Wu, D., Thiagarajan, M., et al. (2006). Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote.PLoS Biol 4(9): e286.

3. Christensen, S. T., Guerra, C. F., Awan, A., Wheatley, D. N., & Satir, P. (2003). Insulin receptor-like proteins in Tetrahymena thermophila ciliary membranes. *Current Biology*, *13*(2), R50-R52.

4. Bartholomew, J., Reichart, J., Mundy, R., Recktenwald, J., Keyser, S., Riddle, M., & Kuruvilla, H. (2008). GTP avoidance in *Tetrahymena thermophila* requires tyrosine kinase activity, intracellular calcium, NOS, and guanylyl cyclase. *Purinergic Signalling*, *4*(2), 171–181.

5. Mace, S. R., Dean, J. G., Murphy, J. R., Rhodes, J. L., & Kuruvilla, H. G. (2000). PACAP-38 is a chemorepellent and an agonist for the lysozyme receptor in *Tetrahymena thermophila*. *Journal of Comparative Physiology A*,*186*(1), 39-43.

6. Robinette, E. D., Gulley, K. T., Cassity, K. J., King, E. E., Nielsen, A. J., Rozelle, C. L., ... & Kuruvilla, H. G. (2008). A comparison of the polycation receptors of Paramecium tetraurelia and *Tetrahymena thermophila. Journal of Eukaryotic Microbiology*, *55*(2), 86-90.

7. 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), 11-17.

Contact Information

Heather G. Kuruvilla, Professor of Biology
heatherkuruvilla@cedarville.edu