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Netrin-1-like Peptides Are Secreted by Tetrahymena thermophila

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Abstract

Netrin-1 is a peptide signaling molecule that has many roles in vertebrates. In the ciliated protozoan, *Tetrahymena thermophila*, netrin-1 acts as a chemorepellent, causing cells to exhibit a characteristic avoidance behavior. We have previously shown that netrin-1 avoidance by *T. thermophila* is inhibited by genistein, which is a broad spectrum tyrosine kinase inhibitor. One question we wished to answer in our current study was, "Is genistein specifically acting upon tyrosine kinases in order to inhibit netrin avoidance in *Tetrahymena*?" In order to answer this question, we used the phytoestrogen, diadzein, as a negative control for genistein inhibition. Diadzein had no effect on avoidance, suggesting that genistein inhibition is specific. In order to gain more information about the nature of the kinases involved in netrin-1 signaling, we tested several other kinase inhibitors, including a *src* inhibitor, a focal adhesion kinase inhibitor, and a Rho kinase inhibitor. Each of these kinases has been implicated in netrin-1 signaling in some vertebrate cell types. However, none of these inhibitors affected *Tetrahymena* avoidance to netrin-1. Finally, we wished to answer the question, "Is netrin-1 actually serving an autocrine signaling role in *Tetrahymena*, or is the peptide merely serving as an agonist for another receptor?" In order to answer this question, we prepared a whole cell extract of *Tetrahymena* using 0.1% SDS. We also washed *Tetrahymena* in our behavioral buffer and allowed them to sit in that buffer for 24 hours. The *Tetrahymena* were centrifuged out of the buffer, and the supernatant, containing the proteins which the *Tetrahymena* had secreted, was kept for ELISA assay. An ELISA, using a polyclonal anti netrin-1 antibody, was run on the whole cell extract and the secreted proteins against a netrin-1 standard curve. Both the secreted proteins and the whole cell extract tested positive for netrin-1 in the ELISA. Further experimentation will allow us to determine the nature of these netrin-like peptides.

Introduction

A number of compounds, including GTP (Bartholomew *et al*. 2008) and PACAP (Mace *et al*., 2000; Keedy *et al*., 2003), are chemorepellents in *Tetrahymena thermophila.* PACAP appears to signal through a G-protein mediated pathway, which includes adenylyl cyclase and phospholipase C (Keedy *et al*., 2003) while GTP signaling is blocked by genistein and by calcium chelators (Bartholomew *et al*. 2008). We have previously determined that netrin-1 peptide is also a chemorepellent in this species and that signaling through netrin-1 is inhibited by genistein, but unaffected by calcium chelators. Because of this, we hypothesized that netrin-1 signaling would not show cross-adaptation to either PACAP or GTP.

Netrin-1 is a pleiotropic signal in vertebrates, and has been implicated in processes ranging from neuronal growth to cell migration and cell adhesion. Netrin-1 can signal through several receptors including DCC and UNC-5, and a number of second messenger pathways are involved in netrin signaling in vertebrates. Using pharmacological inhibitors, we tried to determine whether the same pathways are used in the netrin-1 response of *Tetrahymena thermophila.*

Since netrin-1 acts as a chemorepellent in *Tetrahymena thermophila*, it is possible that cells make and secrete this peptide in order to signal other cells. We tested this hypothesis by obtaining secreted proteins, as well as whole-cell extract, from *Tetrahymena* and using an anti-netrin-1 peptide antibody to perform ELISA. We found that both secreted proteins and whole cell extracts tested positive for netrin-1.

Materials and Methods

Behavioral assays were conducted using a dissection microscope, a 3-well microtiter plate, and a modified Pasteur pipette as described in Mace *et al.*, 2000 and Robinette *et al.,* 2008. Pharmacological inhibition assays were conducted simiilarly to behavioral assays. Cells were washed in buffer, transferred to the inhibitor being tested for 10-15 minutes, and then transferred to a solution containing a mixture of the inhibitor and the chemorepellent being tested. Cell behavior was observed under a dissection microscope, and individual cells were scored as either positive or negative for avoidance behavior. An avoidance response of 20% or less was considered "baseline avoidance" as cells show this level of avoidance when being transferred from one well containing buffer solution into a second well containing the same buffer solution (Mace et al., 2000).

Whole cell extracts were obtained by taking 10 ml of cells in culture, washing them twice in buffer, reconstituting them in 1 ml of buffer, and adding Triton X-100 or SDS to a final concentration of 0.1%. Cells were incubated on ice for 1-2 hours with the addition of a protease inhibitor cocktail. Secreted proteins were obtained by taking 10 ml of cells in culture, washing them twice in buffer, and incubating them overnight in 2 ml of buffer. After 24 hours of incubation, cells were spun down in a clinical centrifuge, and the supernatant was kept for ELISA .

For ELISA, a polyclonal goat anti-netrin-1 peptide antibody was used as the primary antibody at a 1:10,000 dilution. HRP-conjgated rabbit anti-goat antibody was used a secondary antibody at a 1:5,000 dilution. Pierce's Quanta Red was used as substrate. Netrin-1 peptide was used as a positive control.

Results

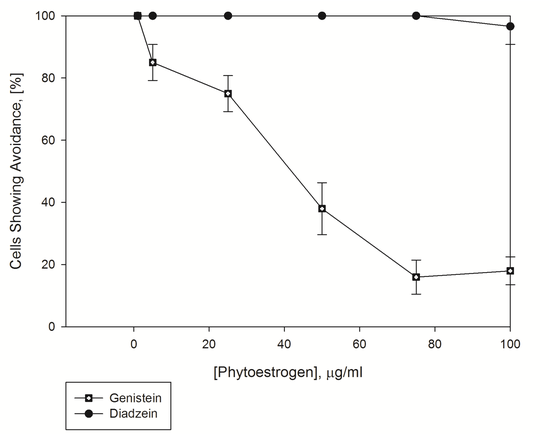

Figure 1. Netrin-1 appears to signal through a tyrosine kinase in *Tetrahymena thermophila.* Genistein (open squares), a broad-spectrum tyrosine kinase inhibitor, reduced avoidance of netrin-1 to baseline levels (< 20%). Daidzein, a phytoestrogen used as a control for side effects of genistein, had no effect on avoidance behavior, indicating that genistein inhibition is specific.

Table 1. Cross-adaptation assays indicate that Netrin-1 uses a different signaling pathway than the previously described PACAP and GTP pathways in *Tetrahymena thermophila.* Shaded boxes indicate controls, where cells are adapted to one peptide and retested in the same peptide. Avoidance in our controls is baseline (less than 20%). However, none of the other chemorepellents showed cross-adaptation, as avoidance remained near 100%.

	GTP Signals through tyrosine kinase and calcium signaling (Bartholomew et al., 2008)	**PACAP** Signals thru G-protein pathway (Keedy et al., 2003)	**Netrin-1** Signals through tyrosine kinase, no calcium involvement (Kuruvillla, unpublished data)
GTP	13.3 ± 5.8	95.0 ± 5.0	93.0 ± 4.1
PACAP	96.67 ± 5.2	12.5 ± 9.6%	95 ± 5.5%
Netrin-1	90 ± 10.0%	90 ± 0%	6.66 ± 5.8%

Table 2. Pharmacological inhibition studies indicate that *Tetrahymena thermophila* do not use calcium, G-proteins, Rho kinases, *src* family kinases, or focal adhesion kinases in signaling through netrin-1. In vertebrate systems, netrin-1 signals through a number of these pathways, depending upon cell type.

Inhibitor	Targets	Inhibition
EGTA	Extracellular calcium	None
BAPTA	Cytosolic calcium	None
Thapsigargin	ER calcium stores	None
GDP-β-S	G-proteins	None
Pertussis Toxin	Gi/o proteins	None
Rp-cAMPs	PKA	None
U73122	PLC	None
Genistein	Tyrosine Kinases	Inhibits Avoidance
Daidzein	Control for Genistein	None
Neomycin	Polycation Receptor	None
SU6668	Protein Kinases	None
NS-2028	Guanylyl Cyclase	None
GSK 429286	Rho Kinase	None
Src I-1	Src family Kinases	None
FAK inhibitor 14	Focal Adhesion Kinase	None

Table 3. ELISA data show that netrin-1 is present in whole-cell extracts as well as secreted proteins obtained from *Tetrahymena thermophila.* Netrin-1 concentrations were estimated from a standard curve of netrin-1 peptide dilutions. Total protein concentrations were estimated from a standard curve of lysozyme concentrations measured at OD_{280}.

Protein Component	Netrin-1 Concentration, µM	Total Protein Concentration, mg/ml
Whole Cell Extract, SDS	0.09	2.5
Whole Cell Extract, Triton X-100	0.05	3.5
Secreted Protein	0.01	1

Conclusions

- Daidzein studies indicate that blocking of netrin-1 signaling is specific, and is likely mediated by a tyrosine kinase.
- Cross-adaptation studies indicate that netrin-1 does not signal through the same pathways as either GTP or PACAP.
- Netrin-1 signaling in *Tetrahymena* is unaffected by *src*, focal adhesion kinase, or Rho kinase.
- ELISA indicates that a netrin-1 like peptide is present both in *Tetrahymena* whole cell extract, as well as in proteins secreted from *Tetrahymena.* This indicates that *Tetrahymena* may be using netrin-1 like peptides to communicate with each other or with other organisms.

References

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.

Csaba, G. 2012. The hormonal system of the unicellular *Tetrahymena*: A review with evolutionary aspects. *Acta Microbiologica et Immunologica Hungarica,* 59 (2):131–156.

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: 11-17.

Mace, S.R., Dean, J.G., Murphy, J.R., Rhodes, J.L, and Kuruvilla, H.G. 2000. PACAP-38 is an agonist for the lysozyme receptor in *Tetrahymena thermophila*, *J. Comp. Physiol. A.,* 186(1):39-43.

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.

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