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The Chemorepellent, Netrin-1, Appears to Signal Through a Tyrosine Kinase in Tetrahymena thermophila

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Abstract

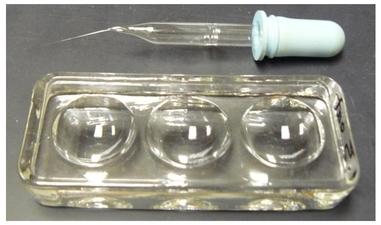
Netrin-1 is a pleiotropic peptide signaling molecule. Its most well-known role in vertebrate development is neuronal guidance. Depending upon the cell type and signal concentration gradient, netrin-1 may serve either as a chemoattractant, causing formation of axonal growth cones, or as a chemorepellent, causing growth cone collapse within the axon. Netrin-1 can bind to at least two types of receptors, and uses a variety of signaling proteins to convey its message. In some vertebrate cell types, the netrin-1 signal is G-protein mediated, while in other cell types, netrin signaling requires a tyrosine kinase or some other combination of kinases in order to signal. *Tetrahymena thermophila* are free-living, eukaryotic cells that can respond to chemoattractants and chemorepellents by moving toward attractants and away from repellents. By studying the behavior of these organisms, we have found that netrin-1 acts as a chemorepellent in *T. thermophila.* Response to netrin-1 is concentration dependent, with an EC_{100} of approximately 1 μM, and an EC_{50} of approximately 10 pM. Netrin-1 avoidance may be effectively eliminated by the addition of the broad-spectrum tyrosine kinase inhibitor, genistein, to the behavioral assay. The IC_{100} of genistein was approximately 75 μg/ml, while the IC_{50} of this compound was near 50 μg/ml. G-protein inhibitors, calcium chelators, and a number of other pharmacological inhibitors had no effect on netrin-1 signaling in this organism. These data show that netrin-1 is a chemorepellent in *Tetrahymena thermophila* and that netrin signaling appears to implicate a tyrosine kinase in this organism. Further studies will help us to determine whether genistein is specifically acting upon a tyrosine kinase pathway or whether the inhibition is occurring via some other genistein-mediated effect.

Introduction

Since the 1970s, *Tetrahymena thermophila* have been used as a model system in which to study hormones, second messenger systems, and chemotaxis (Csaba, 2012). These organisms are easy to grow and maintain on an inexpensive medium, making them an attractive experimental system. In addition, the behavior of *Tetrahymena* toward chemorepellents and chemoattractants is observable under a light microscope. *Tetrahymena* respond to chemoattractants by increasing their swimming speed, which can be timed using video technology. In contrast, they respond to chemorepellents by reversing the direction of their ciliary beat, which disrupts their forward swimming pattern. This causes them to jerk back and forth, swim in small circles, or occasionally "log roll" in place. All of these swimming behaviors may be observed under a dissection microscope. Our previous experiments with *T. thermophila* have shown that netrin-1 is a chemorepellent in this organism at micromolar concentrations. In our current research, we asked the question, "What signaling pathways does netrin-1 use in *Tettrahymenal*? Do *Tetrahymena* signal through the previously described polycation receptor (Keedy *et al.,* 2003) or are they using some other mechanism?

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 as pictured below.



Cell suspension was placed in the first well. A buffer (control) was placed in the second well. The peptide of interest, dissolved in the same buffer, was placed in the third well. Cells were individually picked up and moved from one well to another under a dissection microscope, using the modified Pasteur pipette. Each cell was scored as positive or negative for avoidance. Cells were counted in groups of ten so that average percent avoidance could be calculated.

For inhibitor studies, the assay was similar. The first well contained cell suspension. The second well contained the pharmacological inhibitor of interest. Cells were incubated in this well for 10-15 minutes before being transferred to the third well, which contained the inhibitor along with the peptide of interest. Cells were scored as positive or negative for avoidance, and average percent avoidance was calculated as previously described. The percentage of cells showing avoidance in the presence of the inhibitor was compared to avoidance in the absence of inhibitor.

Results

Figure 1. Amino Acid Sequences of Netrin-1 Peptide used in this study show that it is polycationic at our assay pH of 7.0. Positively charged amino acids are shown in red, while negatively charged amino acids are shown in blue.

Netrin-1 Peptide

KFQQREKKGKCKKA

Net charge = +6 at pH 7.0

Figure 2. Netrin-1 is a chemorepellent effective at micromolar concentrations in *Tetrahymena thermophila.* The EC_{100} of the peptide was approximately 1 μM.

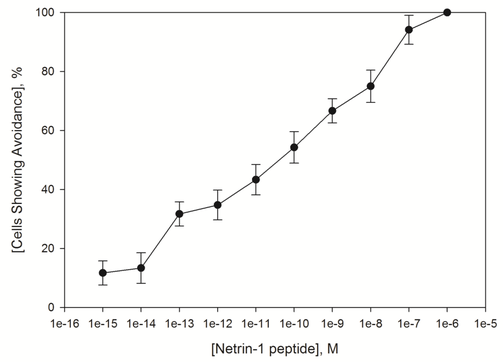


Figure 3. Genistein, a broad-spectrum tyrosine kinase inhibitor, blocks behavioral avoidance to netrin-1 in *Tetrahymena thermophila.*

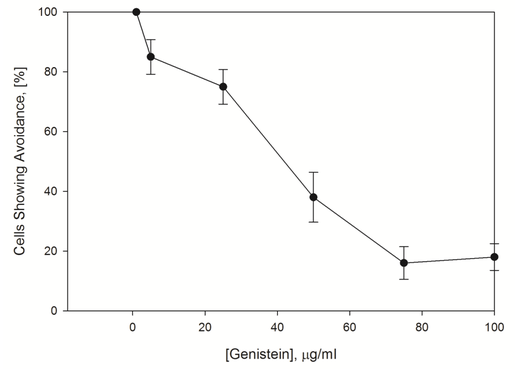


Table 1—Many other pharmacological inhibitors had no effect on avoidance to netrin-1. This implies that these peptides are not signaling through a G-protein coupled receptor or the previously described polycation receptor (Keedy *et al*., 2003). Intracellular and extracellular calcium do not appear to be involved in this response.

Inhibitor Used	Mechanism of Action	Effect on Netrin-1 Avoidance
Pertussis Toxin	Gi/o protein inhibitor	None
EGTA	Extracellular calcium chelator	None
BAPTA-AM	Intracellular calcium chelator	None
Neomycin sulfate	Competitive inhibitor of polycation receptor	None
Thapsigargin	Depletes calcium from ER stores	None
GDP-β-S	Broad spectrum G-protein inhibitor	None
Rp-cAMPs	Analogue of cAMP, inhibits PKA	None
U-73122	Phospholipase C inhibitor	None
SU-6668	Broad spectrum kinase inhibitor	None
NS-2028	Guanylyl cyclase inhibitor	None

Table 2. Cross-adaptation of Netrin-1 Peptide vs. ACTH 6-24 indicates that the two chemorepellents could share some aspect of their signaling pathway.

	Netrin-1 Peptide	ACTH 6-24
Netrin-1 Peptide	16.6 ± 5.8%	0.0 ± 0%
ACTH 6-24	80.0 ± 8.2%	13.3 ± 5.8%

Conclusions

- Netrin-1 peptide is a chemorepellent in *Tetrahymena thermophila* in the micromolar range.
- Avoidance to netrin-1 peptide is blocked by the addition of genistein, suggesting tyrosine kinase involvement.
- Cross-adaptation of Netrin-1 Peptide vs. ACTH 6-24 indicates that the two chemorepellents could share some aspect of their signaling pathway.
- Using diadzein as a negative control, we hope to ascertain whether the genistein-mediated inhibition is specific.

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