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Netrin-1 Signals Through Protein Kinases in *Tetrahymena thermophila*

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Abstract

Netrins are a family of signaling proteins involved in developmental processes such as neuronal guidance and angiogenesis. The best characterized netrin, netrin-1, signals through a number of different receptors. When acting as a chemoattractant, netrin-1 primarily signals through the DCC receptor and associated protein tyrosine kinase and MAP kinase signaling pathways. When acting as a chemorepellent, netrin-1 signals through the UNC5 receptor, which involves recruitment of the protein tyrosine phosphatase, SHP2.

While netrins are ubiquitously expressed throughout the animal kingdom, our laboratory was the first to describe a netrin-1 like protein in *Tetrahymena.* This netrin-1 like protein is secreted from *Tetrahymena* and acts as a chemorepellent. In our current study, we describe signaling through netrin-1 in this organism. Netrin-1 signaling is inhibited by the tyrosine kinase inhibitor, hypericin, and by the broad-spectrum kinase inhibitor, apigenin, both acting in the micromolar range. We are conducting further studies to determine whether netrin-1 signaling results in changes to the phosphorylation state of intracellular proteins.

Introduction

Chemorepellent signaling in neurons via netrin-1 generally involves the UNC-5 receptor, tyrosine kinase signaling, and the eventual recruitment of a tyrosine phosphatase. In our previous study, we demonstrated that *Tetrahymena thermophila* respond to netrin-1 peptide by exhibiting avoidance behavior (Kuruvilla *et al*., 2016). Avoidance may be blocked by addition of the broad spectrum tyrosine kinase inhibitor, genistein, to the reaction buffer. A similar phytoestrogen, diadzein, has no effect on avoidance, indicating that genistein's effects are specific. However, when we used immunofluorescence to probe netrin-1 treated cells with an antiphosphotyrosine antibody, there was no increase in fluorescence in netrin-1 treated cells when compared to controls (Kuruvilla *et al*., 2016). The existence of tyrosine kinases in *Tetrahymena* remains controversial. Genomic studies have not found a tyrosine kinase in *Tetrahymena (Eisen et al.,* 2006)**;** however, tyrosine kinase signaling has been implicated in GTP signaling (Bartholomew *et al*., 2008) as well as in insulin-like receptors (Christensen *et al.*, 2003) in *Tetrahymena*. In our current study, we used pharmacological inhibitors as well as immunofluorescence in order to further assess the netrin-1 signaling pathway in this organism.

Methods and Materials

Behavioral Assays
Behavioral assays were carried out as previously described (Mace *et al*., 2000). Pharmacological inhibition assays were performed similarly to the behavioral assays described above (Keedy *et al*., 2003).

Immunofluorescence
Immunofluorescence was carried out using a modified protocol obtained from cellsignal.com. Briefly, cells were washed twice in PBS, reconstituted in 3.7% formaldehyde in PBS, and fixed for 15 min at room temperature. After fixation, cells were rinsed three times in PBS before being blocked in blocking buffer for 60 minutes. After washing off blocking buffer, 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 fluorochrome-containing 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 covered with a coverslip and observed under a fluorescence microscope at 400X.

Results

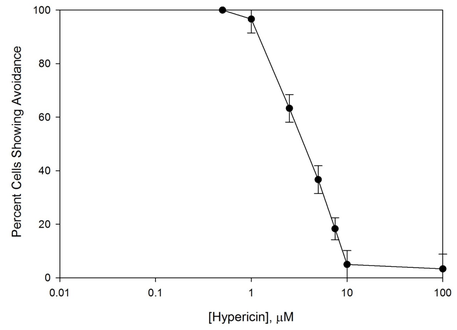

Figure 1. The tyrosine kinase inhibitor, hypericin, inhibits netrin-1 avoidance in *Tetrahymena thermophila.* The IC_{50} of this compound was between 5 and 7 µM.

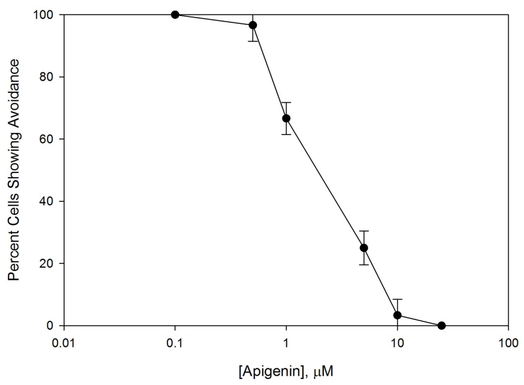

Figure 2. The broad spectrum kinase inhibitor, apigenin, inhibits netrin-1 avoidance in *Tetrahymena thermophila.* The IC_{50} of this compound was between 1and 5 µM.

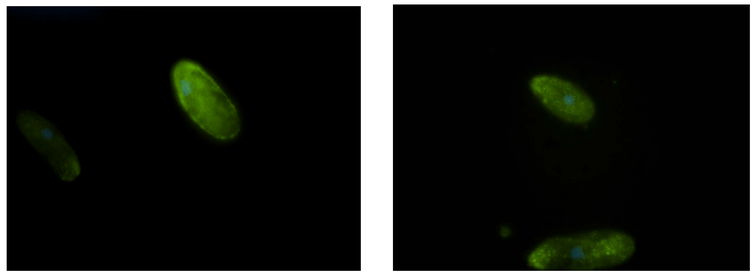

Figure 3. Staining with an anti-phosphoserine antibody shows comparable levels of staining in control cells (left) and netrin-1 treated cells (right). Fluorescence is also distributed similarly throughout the cell in both samples. Green staining indicates phosphoserine; blue staining indicates DAPI.

Conclusions

- Hypericin, a tyrosine kinase inhibitor, inhibits netrin-1 avoidance, supporting the hypothesis that tyrosine kinases are important for netrin-1 signaling in *Tetrahymena.*
- Sodium orthovanadate, a protein tyrosine phosphatase inhibitor, had no effect on avoidance.
- Apigenin, a broad spectrum phosphatase inhibitor, also inhibited netrin-1 avoidance.
- Immunofluorescence using an antiphosphoserine antibody shows similar levels of serine phosphorylation are present in control and netrin-1 treated controls.

Future Experiments

- Repeat phosphotyrosine staining experiment in the presence of tyrosine phosphatase inhibitors in order to determine whether tyrosine phosphorylation occurs when cells are treated with netrin-1.
- Do a Western blot with anti-phosphotyrosine antibodies in the presence and absence of netrin-1 to determine if phosphotyrosine levels increase when netrin-1 signals.
- Use an antibody against UNC-5 to determine whether a homolog of this receptor exists in *Tetrahymena*.

Contact

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