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Netrin-3 Peptide (C-19) is a Chemorepellent and a Growth Inhibitor in *Tetrahymena thermophila*

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Abstract

The netrins are a family of signaling proteins expressed throughout the animal kingdom. Netrins play important roles in developmental processes such as axonal guidance and angiogenesis. Netrin-1, for example, can act as either a chemoattractant or a chemorepellent for axonal growth cones depending upon the concentration of the protein as well as the cell type. Netrin-1 acts as a growth factor in some cell types and is expressed by some tumor cells. Netrin-3 appears to share some signaling apparatus with netrin-1, but is less widely expressed, and its physiological roles are much less understood.

Tetrahymena thermophila are free-living, eukaryotic, ciliated protozoa used as a model system for studying chemorepellents and chemoattractants because their swimming behavior is readily observable under a microscope. We have previously found that netrin-1 peptide acts as a chemorepellent in *Tetrahymena thermophila* at concentrations ranging from micromolar to nanomolar. However, netrin-1 peptide does not affect growth in *Tetrahymena* at these concentrations. In our current study, we have found that related peptides, netrin-3 peptide (H-19 and C-19; Santa Cruz Biotechnology), act as chemorepellents in *Tetrahymena thermophila* at concentrations at or below 1 μg/ml. The same concentration of netrin-3 peptide reduces growth of *Tetrahymena* cultures by approximately 75%. We are currently conducting further studies to determine the mechanism through which these peptides are signaling.

Materials and Methods

Growth Assays
Tetrahymena thermophila, strain B2086.2, were inoculated into the axenic medium of Dentler. To each 2 ml of cell suspension, netrin-3 peptide, H-19 or C-19, was added to a final concentration of 1 μg/ml. The control culture received an equal volume of buffer. Cells were grown under sterile conditions for two days, fixed in formaldehyde, and counted using a Bio-Rad TC10 Automatic Cell Counter. Prior to fixation, an aliquot of cells from each group was tested for viability using propidium iodide staining. A two-tailed T-test was run to determine if results showed a significant difference from control growth.

Cross-Adaptation Assays
Cross-adaptation was carried out using the EC_{100} for each peptide; 1 μM for netrin-1 peptide, 1 μg/ml for netrin 3 (H-19), and 1 μg/ml for netrin-3 (C-19). Cells were exposed to the first peptide, allowed to adapt for 10 minutes, and then transferred to the second peptide. Avoidance behavior was then measured under a dissection microscope. Baseline avoidance (<20%) was seen as evidence that cells used the same signaling pathway to detect both peptides in the assay. High amounts of avoidance (~ 100%) were seen as evidence that the cells used different receptors and/or signaling pathways to detect the two peptides used in the assay.

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 allowed to fix 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.

Western Blotting
Protein extracts were prepared from 2-day old *Tetrahymena* cultures, run on a CM-Sephadex ion exchange column, and run on a 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, and Western blots were performed using a 1:500 dilution of goat anti-netrin-3 (H-19) IgG as the primary antibody and a 1:1000 dilution of rabbit-anti-goat IgG, alkaline phosphatase conjugate, as the secondary antibody. NBT substrate was used to show alkaline phosphatase activity.

Results

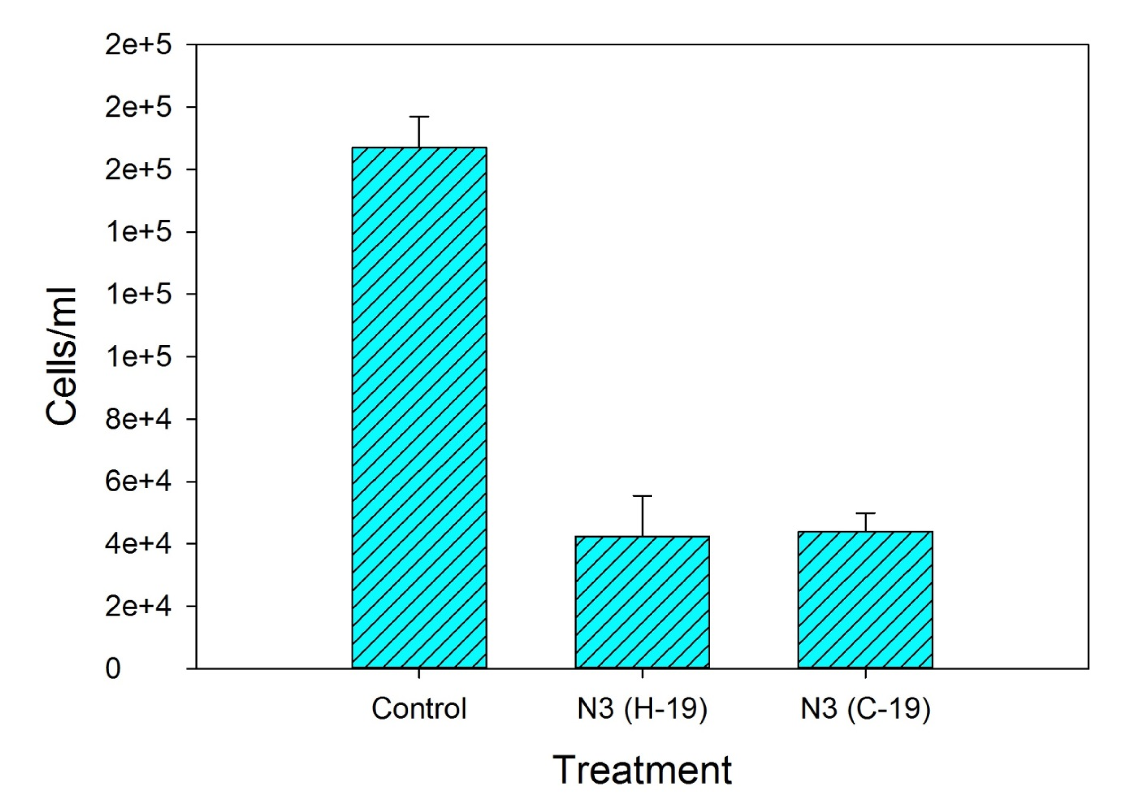

Figure 1. Both N3 (H-19) and N-3 (C-19) significantly decrease the rate of mitosis in *Tetrahymena thermophila* (P = 0.00028 for N3 (H-19) and 0.00022 for N-3 (C-19)). In contrast, netrin-1 peptide does not appear to affect mitotic rate in this organism (data not shown). Viability of N-3 treated cells was assayed using propidium iodide staining. Neither the control group nor either of the netrin-treated groups showed any staining with propidium iodide; in addition, cells in all three groups displayed normal swimming behavior. **This indicates that netrin-3 peptides are acting as growth inhibitors rather than simply killing the cells.**

Table 1. Cross-adaptation indicates that N3 (H-19) and N3 (C-19) cause avoidance in *Tetrahymena thermophila* using the same signaling pathway. In contrast, netrin-1 peptide does not appear to share a signaling pathway with netrin-3 in this organism.

	N3 (H-19)	N3 (C-19)	N1
N3 (H-19)	10.0 ± 0.0	11.66 ± 4.08	96.67 ± 5.16
N3 (C-19)	13.33 ± 5.16	10.0 ± 0.0	96.67 ± 5.16
N1	96.67 ± 5.16	98.33 ± 4.08	13.33 ± 5.16

Figure 2. Immunofluorescence using an anti-N3 (H-19) antibody indicates that an N-3 like protein is present on the cilia of *Tetrahymena*. Top left shows all stains merged, top right shows DAPI, bottom left shows tubulin (green) and bottom right shows N3-reactivity (red).

Figure 3. Western blotting using an anti N-3 (H-19) antibody on fractions obtained by ion exchange chromatography indicate that the N-3 like protein of *Tetrahymena thermophila* is a basic protein with a molecular weight of approximately 48 kD.

Conclusions

- **Both N3(H-19) and N3(C-19) significantly inhibit *Tetrahymena* growth at 1 μg/ml without affecting cell viability.**
- **Both N3(H-19) and N3(C-19) use the same signaling pathway in *Tetrahymena*, but signaling does not appear to overlap with netrin-1 peptide.**
- **An N-3 like protein immunolocalizes to cilia in *Tetrahymena.***
- **The N-3 like protein of *Tetrahymena* is a basic protein with a molecular weight of approximately 48 kD.**

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