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# Recombinant Netrin-4 Does Not Signal Through the Netrin-1 or Netrin-3 Pathway in *Tetrahymena thermophila*

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# Recombinant Netrin-4 does not signal through the Netrin-1 or Netrin-3 Pathway in *Tetrahymena thermophila*

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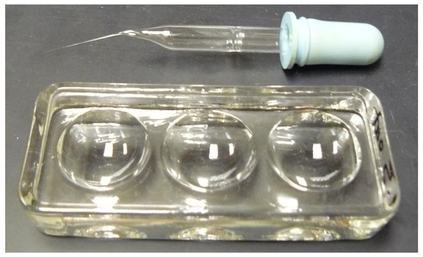
## Abstract and Introduction

Netrin 4 protein and its homologs are found throughout the animal kingdom. Netrin-4 is known to have a protective role against vascular damage. Previous studies have shown that human netrin-1 has a role in angiogenesis. This information about human netrin-1 and netrin-4 led us to research the pathway of netrin-4 in *Tetrahymena thermophila*. Our previous studies of the netrin proteins show that netrin-1 and netrin-3 are both repellents in *Tetrahymena thermophila*. The data in this study show that netrin-4 is also a repellent of *Tetrahymena thermophila*. These data suggest that netrin-4 could signal through the same pathway as netrin-1 and netrin-3. Furthermore, in our previous studies of the signaling pathways for netrin-1 and netrin-3, we discovered that netrin-1 signals through a tyrosine kinase pathway and netrin-3 signals through a serine/threonine kinase pathway. Because of this we decided to investigate whether or not netrin-4 signals through the same or different pathway as netrin-1 or netrin-3. We performed a cross adaptation assay on *Tetrahymena thermophila* using netrin-1, netrin-3, and netrin-4. The data from this assay suggests that netrin-4 does not signal through the same pathway as netrin-1 or netrin-3. Netrin-4 also was shown to have no effect on mitosis, which is similar to previous findings with netrin-1 in this organism. In contrast, netrin-3 peptides have been shown to inhibit mitosis in this organism.

## 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, 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 adaptation studies, the assay was similar. The first well contained cell suspension. The second well contained one of the netrins at its $EC_{100}$. Cells were adapted for 10 minutes before being transferred to the third well, which contained either another netrin at its $EC_{100}$ (test netrin) or the same netrin (controls) Cells were scored as positive or negative for avoidance, and percent avoidance was calculated as previously described. Cells which showed baseline (less than 20%) avoidance were considered cross-adapted.

## Results

```
        10         20         30         40         50
MGSCARLLLL WGCSAVAAGL NGVAGANSRC EKACNPRMGN LALGRKLRAD
        60         70         80         90        100
TMCGQNATEL FCFYSENADL TCRQPKCDKC NAAHSHLAHP PSAMADSSFR
       110        120        130        140        150
FPRTWWQSAE DVHREKIQLD LEAEFYFTHL IMVFKSPRPA AMVLDRSQDF
       160        170        180        190        200
GKTWKPYKYF ATNCSATFGL EDDVVKKGAI CTSRYSNPFP CTGGEVIFRA
       210        220        230        240        250
LSPPYDIENP YSAKVQEQLK ITNLRVRLLK RQSCPCQIND LNAKPHHFMH
       260        270        280        290        300
YAVYDFIVKG SCFCNGHADQ CLPVEGFRPI KAPGAFHVVH GRCMCKHNTA
       310        320        330        340        350
GSHCQHCAPL YNDRPWEAAD GRTGAPNECR TCKCNGHADT CHFDVNVWEA
       360        370        380        390        400
SGNRSGGVCN NCQHNTEGQH CQRCKPGFYR DLRRPFSAPD ACKACSCHPV
       410        420        430        440        450
GSAILPFSSV TFCDPSNGDC PCKPGVAGPH CDRCMVGYWG FGDYGCRPCD
       460        470        480        490        500
CAGSCDPLTG DCISSNADVD WYHEVPAFHS MHNKSEPSWE WEDEQGFSAL
       510        520        530        540        550
RHSGKCECKE QVLGNPKAFC GMKYSYVLKI KILSAHDKGS HAEVNVKIKK
       560        570        580        590        600
VLKSTKLKIL RGKRTLYPES WTNRGCTCPI LNPGLEYLVA GHEDVRTGKL
       610        620
IVNMKSFVQH WKPALGRRVM HILKRDCV
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Figure 1. Primary amino acid sequence of mouse netrin-4 reveals that it is a basic protein. Amino acid sequence was obtained from UniProt. Acidic amino acids are shown in red, basic amino acids are shown in blue. Assuming a pH of 7.0, the charge of this protein is estimated to be +49, with 105 positively charged sidechains and 56 negatively charged sidechains. Since many basic proteins serve as chemorepellents in this organism, we hypothesized that netrin-4 would also be a chemorepellent.

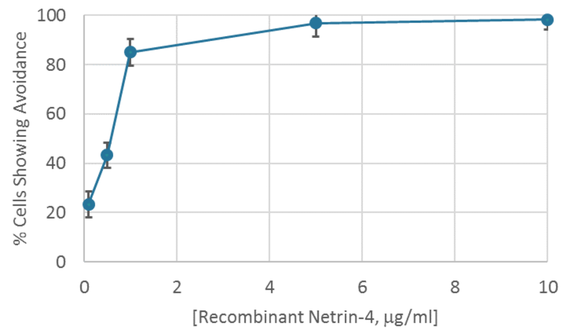


Figure 2. Recombinant mouse netrin-4 protein is a chemorepellent in *Tetrahymena thermophila*. The $EC_{100}$ of this protein is approximately 5 µg/ml, while the $EC_{50}$ is approximately 0.5 µg/ml. Each data point represents the mean ± SD of 6 individual observations.

## Results

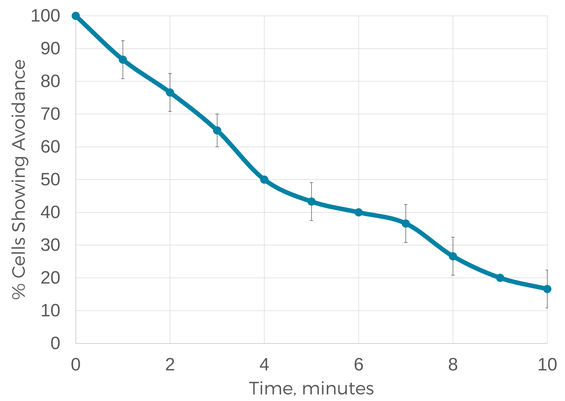


Figure 3. Adaptation to Netrin-4 takes place over a time frame of approximately 10 minutes. As cells become acclimated to the netrin-4, the percentage of avoiding cells gradually declines. Each data point represents the mean ± SD of 6 individual observations.

Table 1. Cross-adaptation assays show no cross-adaptation between any of the three netrins (medium blue boxes) suggesting that the three netrins use different signaling mechanisms in *Tetrahymena*. In contrast, cells adapted to a specific netrin continued to show baseline avoidance upon a second exposure to that netrin (light blue boxes).

| | N1 Peptide | N3N Peptide | N4 recombinant |
|---|---|---|---|
| N1 peptide | 13.33 ± 5.16 | 98.33 ± 4.08 | 98.33 ± 4.08 |
| N3N peptide | 96.67 ± 5.16 | 13.33 ± 5.16 | 100.0 ± 0 |
| N4 recombinant | 96.67 ± 5.16 | 98.33 ± 4.08 | 10.0 ± 0 |

Table 2. Addition of recombinant netrin-4 and netrin-1 peptide to cell cultures has no significant impact on mitotic rate. In contrast. Netrin-3 peptide significantly reduces mitotic rate. All netrins were used at their $EC_{100}$ as determined by behavioral assay.

| | % of Control | Result of two-tailed T-test |
|---|---|---|
| N1 peptide | 98.75 | 0.955 |
| N3 peptide | 55.20 | < 0.000001 |
| N4 recombinant | 95.74 | 0.957 |

## Conclusions

- Netrin-4 is a chemorepellent in *Tetrahymena thermophila*.
- Adaptation to netrin-4 occurs over a ten-minute period, similar to other chemorepellents.
- Netrin-1, 3, and 4 appear to signal through different mechanisms in this organism.
- Netrin-4, like netrin-1, has no significant effect on mitosis in *Tetrahymena thermophila*.

## References

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