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Vertebrate Neuronal Chemorepellents, Semaphorin 3C and Netrin-1, Are Chemorepellents in Tetrahymena Thermophila

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Abstract

During vertebrate development, neuronal growth is guided by chemical signals. Chemoattractants encourage neuronal growth cones to form, while chemorepellents cause growth cone collapse. Integration of these signals allows for proper neural positioning in the developing organism. Not all signaling pathways are clear-cut, however. Netrin-1, for example, can function as either a chemoattractant or a chemorepellent, depending upon the cell type involved, the signals the cell has previously received, and the concentration of the signal. Netrin-1 primarily signals through a G-protein mediated receptor via the adenylyl cyclase pathway. The semaphorins mainly serve as chemorepellents and as immune signals in vertebrates. However, signaling through the semaphorins is not well understood. *Tetrahymena thermophila* are free-living, ciliated eukaryotic organisms that are often used as a model for showing behavioral responses to both chemoattractants and chemorepellents. A number of polycationic peptides function as chemorepellents in this organism, including lysozyme, VIP, PACAP, nociceptin, substance P, and ACTH derivatives. In the current study, we used two polycationic peptides derived from the vertebrate neuronal chemorepellents, semaphorin 3C and netrin-1, in order to determine whether they were chemorepellents in *Tetrahymena thermophila.* Both peptides were chemorepellents in *Tetrahymena thermophila.* Semaphorin 3C peptide showed chemorepellent activity with an EC_{100} of approximately 10 μM. Netrin-1 peptide showed chemorepellent activity over a range of concentrations from micromolar to nanomolar. However, there was a great deal of variability in the response to netrin-1. Further characterization of the pathways involved in the signaling of these repellents will allow comparison between ciliate and vertebrate signaling, and may help us better understand vertebrate development.

Introduction

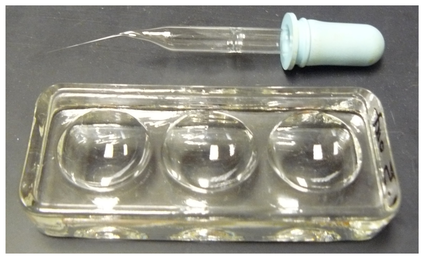
Since the 1970s, *Tetrahymena thermophila* have been used as a model system in which to study hormones, second messenger systems, and chemotaxis (Csaba, 2012). These organisms are easy to grow and maintain on an inexpensive medium, making them an attractive experimental system. In addition, the behavior 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.

Chemorepellent activity in *Tetrahymena thermophila* is often correlated with a calcium-based depolarization (Kuruvilla and Hennessey, 1998; Kuruvilla and Hennessey, 1999; unpublished data). In the absence of electrophysiological testing, calcium involvement in chemorepellent signaling may be ascertained by testing for behavioral avoidance both in the presence and in the absence of a calcium chelator, such as EGTA.

Based on the fact that netrin-1 and semaphorin 3C are polycationic and are vertebrate chemorepellents, we hypothesize that both peptides will be chemorepellents in *Tetrahymena thermophila.* We predict that calcium will be involved in the avoidance response, as it is a critical component of chemorepellent signaling 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, 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 Semaphorin 3C Peptide and Netrin-1 Peptide used in this study show that both are 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. We also tested the full-length, recombinant semaphorin 3-C protein, which is has a molecular weight of 82 kD and a net charge of +33. Due to the length of the sequence, we have not shown it here.

Semaphorin 3-C Peptide
CALINSRKSRNRRNQLPES
Net charge = +4 at pH 7.0

Netrin-1 Peptide
KFQQREKKGKCKKA
Net charge = +6 at pH 7.0

Figure 2. Both full-length semaphorin 3C and its associated peptide are chemorepellents in *Tetrahymena thermophila.* Since both the full-length protein and the peptide showed similar dose-response curves, we used the peptide for the duration of our study. The EC_{100} of the peptide was approximately 10 μM.

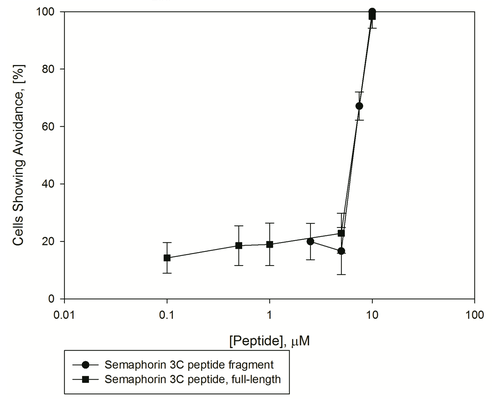


Figure 3. Netrin-1 is a chemorepellent in *Tetrahymena thermophila* over a broad range of concentrations. The variability of the response is extremely high. However, most cells respond well at micromolar concentrations, or at concentrations at or below 10^{-14} M, as indicated by the circles drawn below.

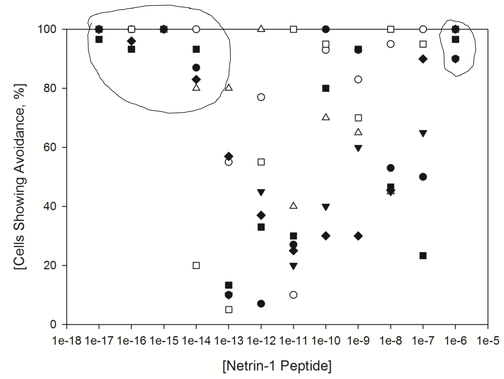


Table 1—Pharmacological Inhibitors had no effect on avoidance to either semaphorin 3C, or netrin-1. This implies that these peptides are not signaling through a G-protein coupled receptor or the previously described polycation receptor (Keedy *et al.*, 2003). Intracellular and extracellular calcium do not appear to be involved in this response.

Inhibitor Used	Mechanism of Action	Effect on Semaphorin 3C Avoidance	Effect on Netrin-1 Avoidance
Pertussis Toxin	Gi/o protein inhibitor	None	None
EGTA	Extracellular calcium chelator	None	None
BAPTA-AM	Intracellular calcium chelator	None	None
Neomycin sulfate	Competitive inhibitor of polycation receptor	None	None

Conclusions

- Semaphorin 3C peptide and netrin-1 peptide are both chemorepellents in *Tetrahymena thermophila.*
- Netrin 1 appears to cause avoidance at both high (micromolar) and low (10^{-14} to 10^{-17}M) concentrations. However, further experiments are needed since the variability in our data cast doubt on its reliability.
- Contrary to our hypothesis, calcium does not appear to be required for signaling through either semaphorin 3C or netrin-1.
- Unlike in humans, G-proteins do not appear to be involved in semaphorin 3C signaling.
- Further research is required in order to determine signaling mechanisms in both pathways.

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