Netrin-1 Signaling in *Tetrahymena thermophila*: The Tyrosine Kinase Controversy Continues

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Netrin-1 Signaling in *Tetrahymena thermophila*: The Tyrosine Kinase Controversy Continues

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**Abstract**

Netrin-1 is a pleiotropic signaling molecule first discovered for its role in neuronal development, where it is largely responsible for axonal guidance. When signaling through the DCC receptor, netrin-1 serves as a chemorepellant; however, when signaling through the UNC receptor results in chemorepulsive activity (Trinh et al., 2012). Our current data imply that the tyrosine kinases are required for signaling in this organism. Our biochemical studies (Christensen et al., 2003; Bartholomew et al., 2008) have suggested that tyrosine kinases are required for netrin signaling. The localization of the staining was similar in both groups. Netrin family of proteins are highly conserved pleiotropic signaling molecules which belong to the laminin superfamily. The netrin family of proteins are highly conserved pleiotropic signaling molecules which belong to the laminin superfamily.

**Materials & Methods**

**Behavioral Assays**

Behavioral assays were carried out as previously described (Mace et al., 2000; Robinette et al., 2008). Adaptation and cross-adaptation assays were carried out as previously described (Keedy et al., 2003). Pharmacological inhibition assays were performed similarly to the behavioral assays described above (Keedy, 2003).

**Immunofluorescence**

*T. thermophila* were washed 3 times in behavioral buffer and then fixed in 3.7% formaldehyde, diluted in behavioral buffer, for 15 minutes. Cells were then washed 3 times in PBS and incubated in blocking buffer overnight. Cells were then washed in PBS and incubated in a 1:100 dilution of either anti-netrin-1 or anti-tubulin antibody in the presence of antibody dilution buffer, for 2 hours with constant shaking. Cells were then once again washed in PBS and then incubated with a 1:100 dilution of secondary antibody, for one hour with constant shaking. Cells were then washed in PBS, stained with DAPI, and viewed under a Nikon H500S Microscope using the Nikon Intensilight C-HGFI. Fluorescence images were obtained with a QI Click 74-0083-04-A camera using NIS Elements BR 4.1.04 Software.

**Results**

**Figure 1. Netrin-1 peptide is a chemorepellent in Tetrahymena thermophila.** The *EC₅₀* of this peptide is 1 μM. The *EC₅₀* of this peptide is approximately 1 nM. "Cells Showing Avoidance" represents the mean of at least 6 trials, and error bars represent the standard deviation. Each trial consisted of 10 cells which were individually observed and scored for avoidance.

**Figure 2. Time course of adaptation to netrin-1 peptide in Tetrahymena thermophila.** Adaptation studies were done at 1 μM netrin-1 peptide, which is the *EC₅₀* of this peptide. "Cells Showing Avoidance" represents the mean of at least 6 trials, and error bars represent the standard deviation. Each trial consisted of 10 cells which were individually observed and scored for avoidance.

**Table 1. Cells adapted to netrin-1 peptide are not cross-adapted to nociceptin, GTP, or PACAP-38.** Percentage avoidance, as listed below, represents the mean ± standard deviation of at least 6 trials. Each trial consisted of 10 cells which were individually observed and scored for avoidance. Adaptation to the same signal (e.g. GTP adapted to GTP, nociceptin adapted to nociceptin, etc.) were run as controls. Each of these controls showed adaptation, showing less than the baseline avoidance of 20% typically seen in behavioral assays.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>GTP</th>
<th>PACAP-38</th>
<th>Nociceptin</th>
<th>Netrin-1 1 Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Baseline</em></td>
<td>94.0 ± 5.2</td>
<td>95.0 ± 5.0</td>
<td>96.6 ± 5.8</td>
<td>96.6 ± 5.8</td>
</tr>
<tr>
<td><em>Adapted</em></td>
<td>93.0 ± 4.1</td>
<td>95.0 ± 5.5</td>
<td>95.0 ± 5.0</td>
<td>96.6 ± 5.8</td>
</tr>
</tbody>
</table>

**Figure 3. Avoidance of netrin-1 peptide is inhibited by the tyrosine kinase inhibitor, genistein, but not by daidzein, a negative control for genistein activity.** The *IC₅₀* of genistein is approximately 50 μg/mL. Baseline avoidance was achieved at a genistein concentration of 75 μg/mL. Daidzein had no effect on avoidance behavior. Percentages represent the mean ± standard deviation of at least 6 trials. Each trial consisted of 10 cells which were individually observed and scored for avoidance.

**Figure 4. Tyrosine phosphorylation levels are not affected by netrin-1 peptide.** Indirect immunofluorescence using PT-66 anti-phosphotyrosine antibody shows no difference in fluorescence intensity between control (A) and netrin-1 exposed cells (B). This indicates that tyrosine phosphorylation is not required for netrin-1 signaling. In contrast, cells stained with an anti-tubulin antibody (C) show a high level of fluorescence intensity.

**Conclusions**

- Netrin-1 peptide is a chemorepellent in Tetrahymena thermophila, causing the cells to exhibit avoidance reactions.
- Cells adapt to netrin-1 peptide over a time course of approximately 10 minutes, similar to the time course seen for other chemorepellents such as PACAP-38 and GTP.
- Cross-adaptation studies indicate that netrin-1 peptide uses a different signaling mechanism than those used by the chemorepellents GTP, PACAP-38, or nociceptin.
- Avoidance of netrin-1 peptide is blocked by addition of the broad spectrum tyrosine kinase inhibitor, genistein, to the behavioral buffer. However, addition of daidzein to the buffer does not affect avoidance.
- Immunostaining using an anti-phosphotyrosine antibody shows no difference in staining intensity between control cells and cells exposed to netrin-1 peptide, giving no evidence of tyrosine kinase activity in this signaling pathway.
- Further experimentation is needed to determine the mechanism of action of genistein in Tetrahymena thermophila.

**References**


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