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Original Communication

Netrin-3-peptides are chemorepellents and mitotic inhibitors in *Tetrahymena thermophila*

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ABSTRACT

Netrins are a family of guidance proteins involved in developmental signaling as well as maintenance of homeostasis within the adult organism. Netrin-1 is the best characterized of all the netrins and has been linked to cancer, apoptosis, angiogenesis, and immune signaling. However, much less is known about netrin-3, especially its roles outside of development. In this study, we performed behavioral assays that demonstrated the chemorepellent effect of netrin-3-peptides on *Tetrahymena thermophila*. Our pharmacological inhibition assays showed that signaling is dependent on calcium and serine/threonine kinases. We also observed that exposing cells to netrin-3-peptides for two days caused a significant decrease in the mitotic rate. Inhibition of mitosis was rescued by adding calcium chelators or a serine/threonine kinase inhibitor to the culture media alongside the netrin-3-peptides. We also used immunofluorescence and enzyme-linked immunosorbent assay (ELISA) assays to determine that *Tetrahymena* secrete a netrin-3-like protein, suggesting a physiological role for a netrin-3-like signal in keeping cell populations from depleting their resources too rapidly. Finally, we contrasted signaling data from our studies with netrin-3-peptides to our data from our previous studies with netrin-1-peptide, showing that the two peptides use different signaling pathways, resulting in dissimilar physiological consequences for the organism.

KEYWORDS: netrin-3, netrin-1, chemorepellent, serine/threonine kinase, mitosis.

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INTRODUCTION

The netrin family is comprised of a group of laminin-like proteins involved in guidance of branched organs and systems during development of animal systems such as the lung, nervous system, and vascular system [1-3]. Netrin secretion continues throughout the life of the organism, with homeostatic roles in the immune system [3, 4] as well as in angiogenesis [2]. In mammals, the family is comprised of netrin-1, netrin-3, netrin-4, netrin G1, and netrin G2 [5]. Netrin-1 is the most extensively studied member of the netrin family. It has a well-characterized role in central nervous system (CNS) development and can act as either a chemoattractant or a chemorepellent [6, 7] depending upon which receptors it binds to. In addition, netrin-1 plays a role as a survival signal [8] and as a biomarker for cancer [9].

In contrast, netrin-3 has been much less studied. Its role in neuronal development is similar to that of netrin-1. However, netrin-3 is more highly expressed in the peripheral nervous system (PNS), while netrin-1 is more highly expressed in the central nervous system (CNS) [10-12]. Also, while netrin-3 is known to signal through netrin-1 receptors, including UNC-5, neogenin, and DCC,

it has a much lower affinity for DCC than netrin-1 [11-13]. Netrin-3 is expressed in root ganglia and limb buds, and has been implicated in embryonic tooth development and myogenic differentiation [10, 14]. Little research has explored the role of netrin-3 outside of these developmental roles.

Previously, our lab discovered that netrin-1-peptide is a chemorepellent in the free-living, ciliated protozoan, *Tetrahymena thermophila* [15]. Signaling appears to be calcium-independent and involves a tyrosine kinase [15]. *Tetrahymena* secrete a netrin-1 like protein, as determined by enzyme-linked immunosorbent assay (ELISA) [15] and Western blotting [15, 16]. In our most recent study, we have been studying the effect of netrin-3 peptides in this organism. We also compared signaling though netrin-3-peptides to what we had previously discovered regarding signaling through netrin-1 peptide in this organism [15, 16].

Our current study demonstrates that netrin-3-peptides are chemorepellents and mitotic inhibitors in *Tetrahymena*, acting through a pathway that is distinct from what has previously been described for netrin-1-peptide. We have also found evidence that *Tetrahymena* secrete a netrin-3-like protein, which may serve a physiological role in conserving resources by slowing population growth.

MATERIALS AND METHODS

Cell cultures

Tetrahymena thermophila, strain B2086.2, were obtained from the Tetrahymena Stock Center at Cornell University (https://tetrahymena.vet.cornell.edu/). Cells were grown at 20 °C in the medium of Dentler without shaking or addition of antibiotics [17]. One day old cell cultures were used for all behavioral and pharmacological assays.

Chemicals and solutions

Netrin-3 peptides (H-13 and C-19), and anti-netrin-3 antibodies (H-13 and C-19) were obtained from Santa Cruz Biotechnology, Dallas TX. ER Tracker™ and Pierce QuantaRed™ Substrate were obtained from ThermoFisher Scientific, Waltham, MA. Antiphosphoserine antibody and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Phosphate buffered saline (PBS) was made by dilution of a 10X stock with distilled water. Behavioral buffer consisted of 10 mM Trizma Base, 0.5 mM MOPS, 50 μM $CaCl_2$; pH was adjusted to 7.0 with 1 mM hydrochloric acid.

Behavioral assays

Behavioral assays were carried out as previously described [15, 18]. *Tetrahymena thermophila* were washed 3 times in behavioral buffer and then 300 μl of cell suspension was transferred to the first well of a microtiter plate. Cells were then transferred individually using a micropipette into the second well of the microtiter plate, already containing 300 μl of buffer as a control. Cells were individually transferred to a third well containing 300 μl of the peptide being tested for chemorepellent activity. Behavior of the individual cells was observed for the first few seconds after they were added to the well, and the percentage of cells exhibiting avoidance behavior was noted.

Pharmacological assays

Pharmacological assays were carried out as previously described [15, 18]. *Tetrahymena thermophila* were washed 3 times in behavioral buffer and then 300 μl of cell suspension was transferred to the first well of a microtiter plate. Cells were then transferred individually using a micropipette into the second well of the microtiter plate, containing 300 μl of a pharmacological inhibitor, and incubated for 15 minutes to allow the cells to take up the inhibitor. Treated cells were individually transferred to a third well containing 300 μl of the peptide being tested for chemorepellent activity in the presence of the pharmacological inhibitor. Behavior of the individual cells was observed for the first few seconds after they were added to the well, and the percentage of cells exhibiting avoidance behavior was noted.

Immunofluorescence

Cells from 2-day old cultures were washed twice in behavioral buffer, reconstituted in 3.7% formaldehyde in behavioral buffer, and fixed for 15 min at room temperature. After fixation, cells were rinsed three times in PBS before incubating in blocking buffer (PBS containing 0.3% Triton

X-100 and 3% bovine serum albumin (BSA) for 60 minutes. After rinsing with PBS, cells were incubated overnight at room temperature in primary antibody at a dilution of 1:100 in the presence of antibody dilution buffer (PBS containing 0.3% Triton X-100 and 1% BSA). After rinsing three times in PBS, cells were incubated with a 1:100 dilution of fluorochrome-conjugated secondary antibody for 1-2 hours at room temperature in the dark, in the presence of antibody dilution buffer. Cells were then rinsed three times in PBS. 10 µl of cell suspension was then applied to a slide and mixed with 10 µl 4′,6-diamidino-2-phenylindole (DAPI). Cell suspension was then covered with a coverslip and observed under a Nikon fluorescence microscope at 400X. Photographs were taken with a QI Click camera using NIS Elements Software, version 4.40. Fluorescence was quantitated using NIS Analysis software, version 4.40.

ELISA

Cell extracts were prepared for ELISA as follows: 2-day old cell cultures were harvested and washed twice in behavioral buffer. Cell pellets were extracted on ice for 60 minutes in 0.5% Triton X-100 in the presence of protease inhibitor cocktail. After extraction, mixture was centrifuged at 4 °C in a microcentrifuge for 15 minutes at 12,500 rpm. Pellets were discarded; supernatants were used for ELISA. Wells were blocked for 1 hour with 1% BSA in PBS-Tween. ELISAs were performed using a 1:1000 dilution of goat anti-netrin-3 or anti-phosphoserine IgG as the primary antibody and a 1:5000 dilution of rabbit-anti-goat IgG, HRP-conjugate, as the secondary antibody. Pierce Quanta-Red™ substrate was used to show horseradish peroxidase (HRP) activity. Fluorescence was read in a Promega GloMax™ Multi Plate Reader.

Growth assays

A newly inoculated *Tetrahymena* culture was aliquotted into sterile 15 ml tubes; 2 ml culture per 15 ml tube to allow for adequate oxygenation. Netrin-3 peptide and pharmacological inhibitors were added to the cultures under sterile conditions and mixed by vortexing. Cells were allowed to grow at 20 °C for 48 hours before they were fixed in 3.7% formaldehyde. Cells were counted in a BIO-RAD TC10™ automated cell counter and counts were manually confirmed using a hemocytometer.

RESULTS

As seen in Figure 1, peptides derived from the N-terminal (N3N) and C-terminal (N3C) ends of netrin-3 act as chemorepellents in *Tetrahymena*. When placed in a solution containing netrin-3 peptides, *Tetrahymena* exhibit avoidance behavior, characterized by jerking, spinning in circles, and trying to reorient themselves. The EC_{100} for both N3N and N3C was 1 µg/ml (Figure 1). The EC_{50} values for the two peptides were also comparable; the EC_{50} for N3N was approximately 0.4 µg/ml, compared with 0.5 µg/ml for N3C (Figure 1).

To establish whether the N3N and N3C peptides and netrin-1-peptide share a common signaling pathway, we performed cross-adaptation assays. When placed in a solution of a chemorepellent for several minutes, *Tetrahymena* cease to show avoidance to this repellent. Peptides that share a pathway exhibit cross-adaptation, indicated by low levels of avoidance by the cells when added to the second peptide. Peptides that do not share a pathway do not exhibit cross-adaptation, indicated by high levels of avoidance by the cells when added to the second peptide. As seen in Table 1, N3N and N3C exhibit cross adaptation, characterized by showing only baseline avoidance, which is considered to be less than 20% [19]. These data suggest that N3N and N3C signal through the same pathway in this organism. Since both peptides appear to signal through the same pathway, we used 1 µM N3N for all subsequent pharmacological assays as well as many of our mitotic assays. In contrast, neither N3N nor N3C exhibits cross adaptation with netrin-1-peptide as indicated by the high levels of avoidance in the cross-adaptation assay, suggesting that netrin-3-peptides and netrin-1-peptides use different signaling pathways in *Tetrahymena* (Table 1).

To further characterize the pathways involved in signaling through netrin-3-peptides, we performed several pharmacological inhibition assays, testing which drugs blocked the chemorepellent response. As seen in Figure 2, both EGTA, an extracellular calcium chelator, and BAPTA-AM, an intracellular calcium chelator, blocked avoidance to N3N. The IC_{50} for EGTA was approximately 35 µM when extrapolating from the curve; however, inhibition appeared to follow more of an “all-or-none” pattern than a typical dose-response curve. A concentration

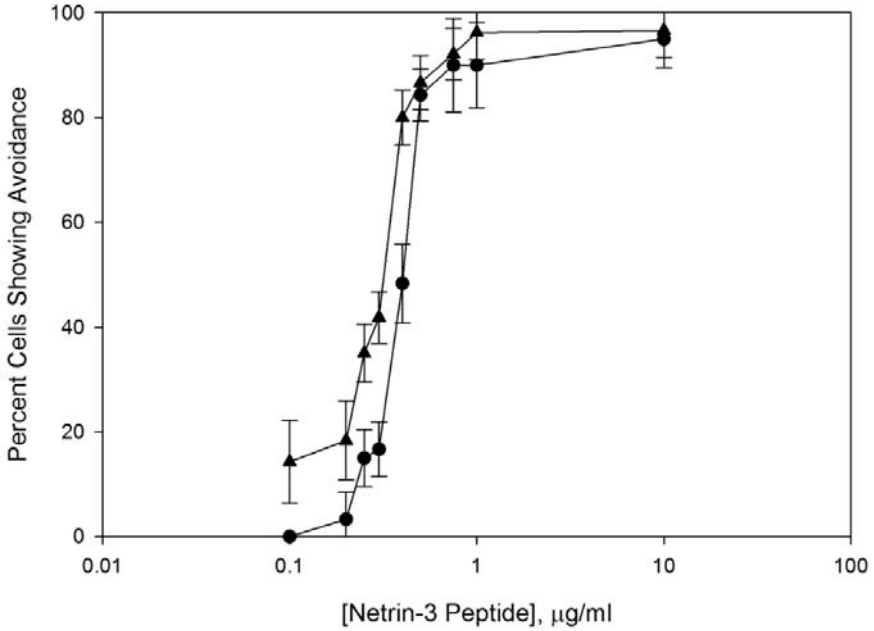

Figure 1. Netrin-3 peptides are chemorepellents in *Tetrahymena thermophila*. A peptide derived from the N-terminal end of human netrin-3 (N3N; closed circles) as well as a peptide derived from the C-terminal tail of mouse netrin-3 (N3C; closed triangles) caused avoidance reactions in *Tetrahymena* in a concentration-dependent manner. The EC_{100} (concentration at which 100% of cells showed avoidance) was approximately 1 µg/ml for both peptides. This concentration was used in subsequent pharmacological assays. The EC_{50} for N3N was approximately 0.4 µg/ml; the EC_{50} for N3C was approximately 0.5 µg/ml. Each trial consisted of 10 cells; each point represents the mean ± standard deviation of 6 trials.

Table 1. Cross-adaptation suggests that peptides derived from the N-terminal end of human netrin-3 (N3N) and a peptide derived from the C-terminal end of mouse netrin-3 (N3C) signal through the same pathway in *Tetrahymena thermophila*. Values ≤ 20% represent cross-adaptation [18, 19]. Values represented in **bold** font represent cross-adapted controls; values in *italic* font represent cross-adaptation between two distinct peptides. In contrast, to the nerin-3-peptides, a peptide derived from human netrin-1 does not cause cross-adaptation with netrin-3 peptides, suggesting that netrin-1 and netrin-3 use different signaling pathways in this organism. Each trial consisted of 10 cells; each value represents the mean ± standard deviation of 6 trials. Since N3N and N3C appear to share a signaling pathway, 1 µM N3N was used in the subsequent pharmacological assays.

	N3N peptide	**N3C peptide**	**N1 peptide**
N3N peptide	**10.0 ± 0.0**	*11.66 ± 4.08*	96.67 ± 5.16
N3C peptide	*13.33 ± 5.16*	**10.0 ± 0.0**	96.67 ± 5.16
N1 peptide	96.67 ± 5.16	98.33 ± 4.08	**13.33 ± 5.16**

of 50 µM EGTA decreased the N3N to baseline avoidance (Figure 2), which is considered to be less than 20% [19]. Similarly, 5 µM BAPTA-AM was sufficient to reduce avoidance to N3N to baseline levels (Figure 2); the extrapolated IC_{50} of this compound was approximately 0.75 µM. In addition, apigenin, a serine/threonine kinase inhibitor, also blocked avoidance to N3N (Figure 3) with an IC_{50} of 0.1 µM. Baseline avoidance to N3N was achieved at 1 µM apigenin.

Since apigenin blocked the chemorepellent response, we further explored the possibility that serine

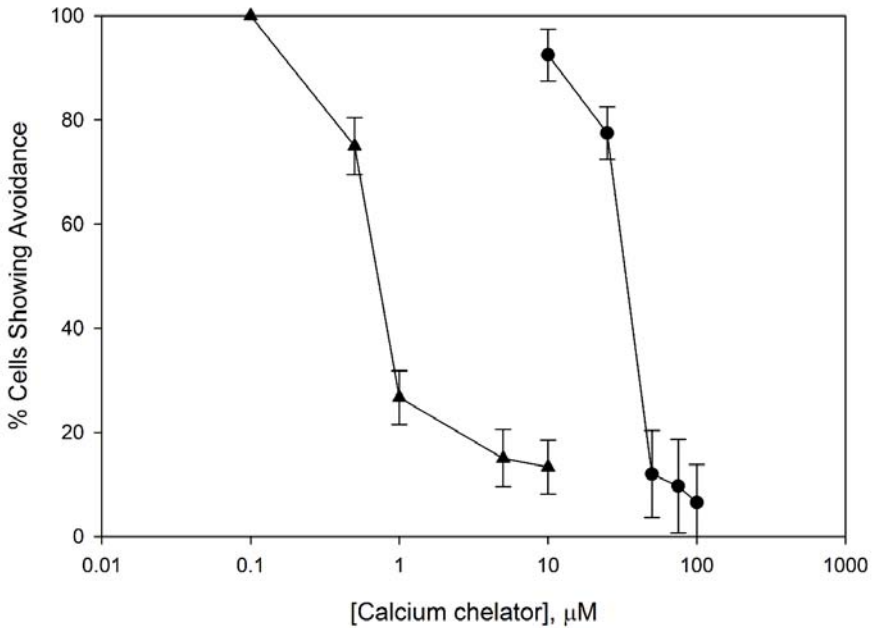

Figure 2. Calcium chelators inhibit avoidance of netrin-3 peptides in *Tetrahymena thermophila.* EGTA, an extracellular calcium chelator (closed circles), and BAPTA-AM, an intracellular calcium chelator (closed triangles), both inhibit avoidance of *Tetrahymena thermophila* when used in the micromolar range. The IC_{50} (concentration at which 50% of avoidance is inhibited) for EGTA was approximately 35 μM, and for BAPTA-AM was approximately 0.75 μM. Each trial consisted of 10 cells; each point represents the mean ± standard deviation of 6 trials.

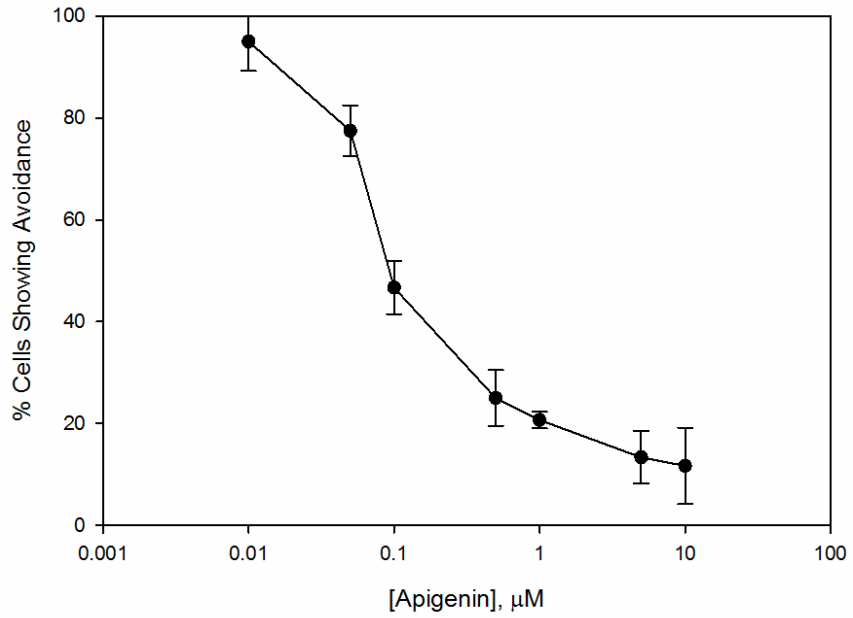

Figure 3. Avoidance of the N-terminal Netrin-3-peptide (N3N) in *Tetrahymena* is inhibited by the serine/ threonine kinase inhibitor, apigenin. The IC_{50} (concentration at which 50% of avoidance is inhibited) for apigenin was approximately 0.1 μM. Each trial consisted of 10 cells; each point represents the mean ± standard deviation of 6 trials.

phosphorylation is involved in netrin-3 signaling. First, we performed ELISA assays to compare serine phosphorylation levels of cells treated with netrin-3-peptide to untreated controls. As seen in Table 2, both netrin-3 peptides, N3N and N3C, approximately doubled serine phosphorylation, a significant increase when compared to control levels of phosphorylation in a two-tailed T-test ($P < 0.005$). Second, we performed immuno-fluorescence to visualize the levels of phosphorylation across the cell. As seen in Figure 4, N3N-treated cells showed significantly higher fluorescence than control cells. The mean fluorescence of the control cells was 59.32 ± 9.16 and the mean fluorescence of the N3N-treated cells was 143.15 ± 27.12. The difference in mean fluorescence gave a P value of < 0.001 when compared in a two-tailed T test.

In addition to its chemorepellent activity, netrin-3-peptides also inhibited mitosis in *Tetrahymena*. As seen in Figure 5, adding 1 μg/ml N3N to a culture of *Tetrahymena* and allowing cells to grow for two days result in a significantly decreased number of *Tetrahymena* cells in culture compared with untreated controls when analyzed in a two-tailed T-test ($P < 0.001$). Mitotic inhibition obtained using N3C was not significantly different from that obtained with N3N (data not shown). In contrast, netrin-1-peptide had no significant effect on mitosis (data not shown; P value = 0.84). The viability of cells treated with netrin-3-peptides, netrin-1-peptides, and pharmacological inhibitors was demonstrated by observing cell motility under a light microscope and by propidium iodide staining (data not shown), indicating that any changes in cell number were caused by a change in mitotic rate rather than the toxicity of the peptides or drugs.

To determine whether the signaling pathways used in mitotic inhibition were similar to those used in avoidance, we added 1 μM apigenin, 5 μM BAPTA-AM, or 50 μM EGTA to cell cultures grown in the presence of 1 μg/ml N3N (Figure 5). Addition of these concentrations of drugs alone showed no significant impact on cell growth (data not shown). Mitosis was rescued by addition of 50 μM EGTA, 10 μM BAPTA-AM, or 10 μM apigenin to the media in the presence of 1 μg/ml netrin-3-peptide (N3N). Apigenin and BAPTA-AM fully rescued mitosis; means were not significantly different from controls in a two-tailed T-test (P values of 0.60 and 0.79 respectively). EGTA partially rescued mitosis; however, the cell count was still significantly lower than the control count ($P < 0.005$). EGTA-treated cell counts were significantly higher than those obtained with N3N alone ($P < 0.001$).

After establishing netrin-3 as a mitotic inhibitor, we investigated whether netrin-3 affected progression through the cell cycle. Since nuclei of cells that are in G_2 have replicated their DNA, resulting in larger nuclear radii, we used DAPI staining of the nucleus to compare nuclear diameter in control and netrin-3 treated cells. As seen in Table 3, N3N-treated cells had significantly larger nuclear radii when compared to control nuclei in a two-tailed T test ($P < 0.001$), suggesting that more N3N-treated cells are in G_2 phase compared to cells growing in the control culture.

In addition to exploring the activity of exogenous netrin-3 peptides as chemorepellents and mitotic

Table 2. Netrin-3 peptides significantly increase serine phosphorylation levels in *Tetrahymena thermophila* as determined by ELISA. Fluorescence unit values represent the mean + standard deviations of three measurements. Relative fluorescence was obtained by comparing netrin-3-treated groups with the control. Fluorescence of each treatment group relative to the control was compared *via* two-tailed T-test. In both cases, P values were < 0.005.

Treatment	Fluorescence units	Relative fluorescence
Control	25,176.67 ± 3060.88	1
N3N	51,420.00 ± 560.83	2.04
N3C	53,233.33 ± 4942.16	2.11

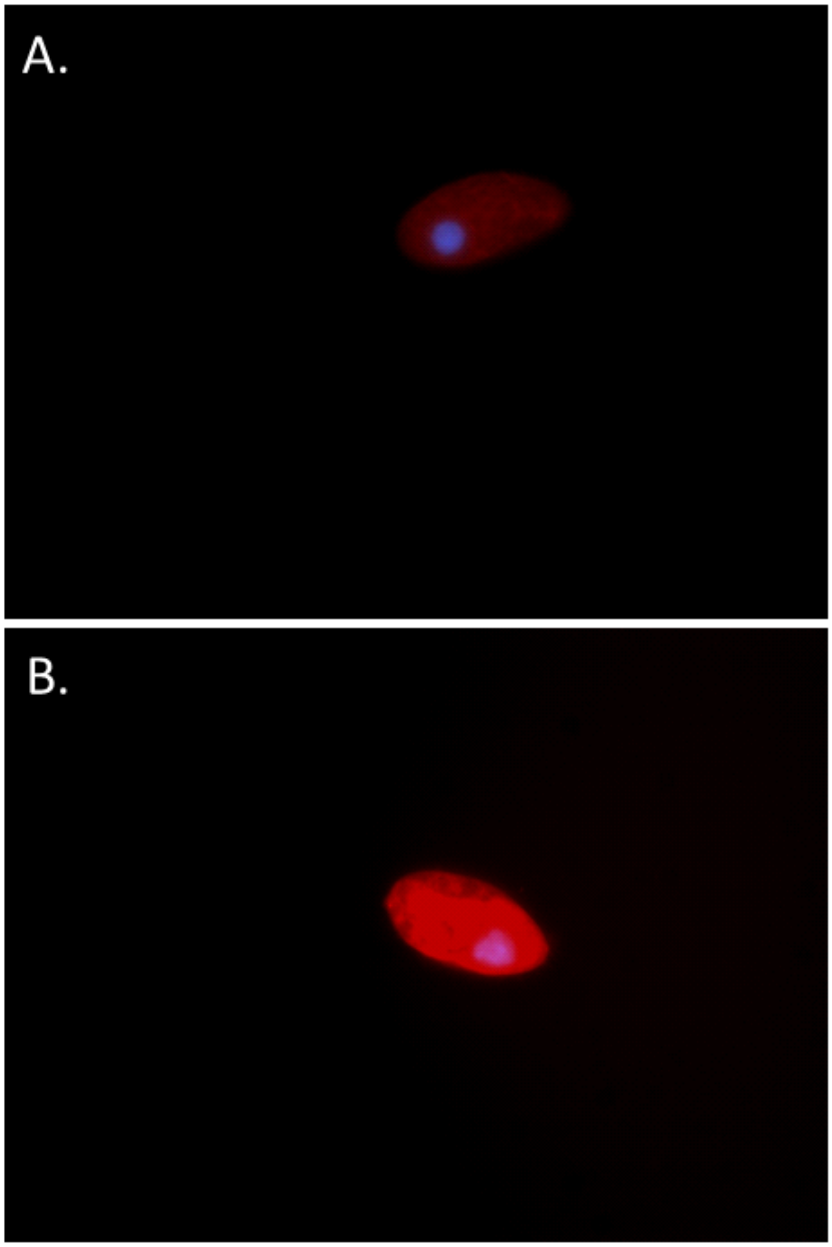

Figure 4. Netrin-3-peptides increase cytosolic serine phosphorylation levels in *Tetrahymena thermophila.* Netrin-3-treated cells (B) showed significantly higher fluorescence than control cells (A). When quantitated, the mean fluorescence of the control cells was 59.32 ± 9.16 and the mean fluorescence of the N3N-treated cells was 143.15 ± 27.12. The difference in mean fluorescence gave a P value of < 0.001 when compared in a two-tailed T test (N = 10 for each sample quantitated). Blue indicates DAPI stain; red indicates staining with the anti-phosphoserine antibody.

inhibitors, we identified a netrin-3-like protein secreted from *Tetrahymena* through ELISA using an anti-netrin-3 antibody. As seen in Figure 6, the concentration of secreted netrin-3-like protein increased as the cell count increased.

Finally, we compared the pharmacological inhibition data from our current study to the data from our previously published work on netrin-1-peptide. As seen in Table 4, netrin-1-peptide and netrin-3-like peptides appear to signal through distinct pathways.

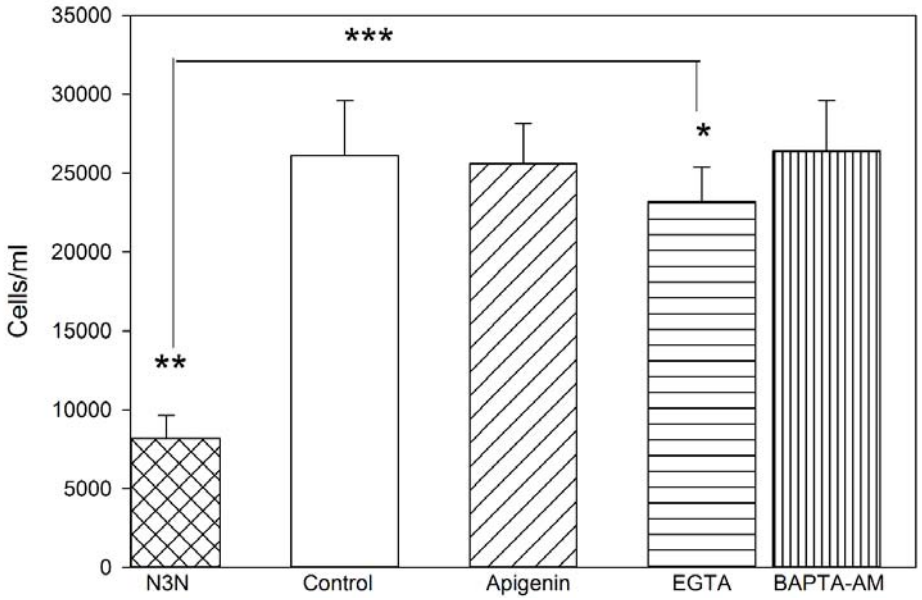

Figure 5. Mitotic inhibition *via* netrin-3 peptide is dependent upon calcium and serine/threonine kinase activity. Mitosis was rescued by addition of 50 μM EGTA, 10 μM BAPTA-AM, or 10 μM apigenin to the media in the presence of 1 μg/ml netrin-3-peptide (N3N). N3N significantly decreased cell number when compared to the control value, when compared *via* two-tailed T-test (**P < 0.001). Apigenin and BAPTA-AM fully rescued mitosis; means were not significantly different from controls in a two-tailed T-test. EGTA partially rescued mitosis; however, the cell count was still significantly lower than the control count (*P < 0.05). EGTA-treated cell counts were significantly higher than those obtained with N3N alone (***P < 0.001). Each bar represents the mean ± standard deviation of at least sixteen measurements.

Table 3. *Tetrahymena* treated with netrin-3-peptide have significantly larger nuclear size, suggesting that cells are arrested in the G2 phase of the cell cycle. Number of control cells measured was 53; number of netrin-3 cells measured was 59. A two-tailed T-test comparing the means of the control and netrin-3 treated cells gave a P-value of < 0.001.

	Control cells	**Netrin-3-peptide-treated cells**
Mean Nuclear Radius, μM	5.16 ± 0.77	5.72 ± 0.73

Signaling through netrin-1-peptide is calcium independent and involves a tyrosine kinase, while signaling through netrin-3-peptides is calcium dependent and involves a serine/threonine kinase. In addition, netrin-1-peptides do not affect mitosis, while netrin-3-peptides reduce mitotic rate.

DISCUSSION

Since very little research has previously characterized netrin-3 outside nervous system development, this study sought to investigate the effects of netrin-3 on *Tetrahymena thermophila* and compare netrin-3 signaling with previously described netrin-1 signaling in this organism. We established that netrin-3 peptides are chemorepellents in *Tetrahymena* as demonstrated by the behavioral assays (Figure 1). The peptides were chemorepellents at low concentrations, with an EC_{100} of 1 μg/ml for both the N3N and N3C peptide. Since the peptide sequences were proprietary, we could not determine the charge of the respective peptides.

Our cross adaptation studies suggest that both N3N and N3C signal through the same pathway (Table 1). In our behavioral assays, *Tetrahymena thermophila* exhibit baseline avoidance, even when transferred to behavioral buffer, of 0-20% [19].

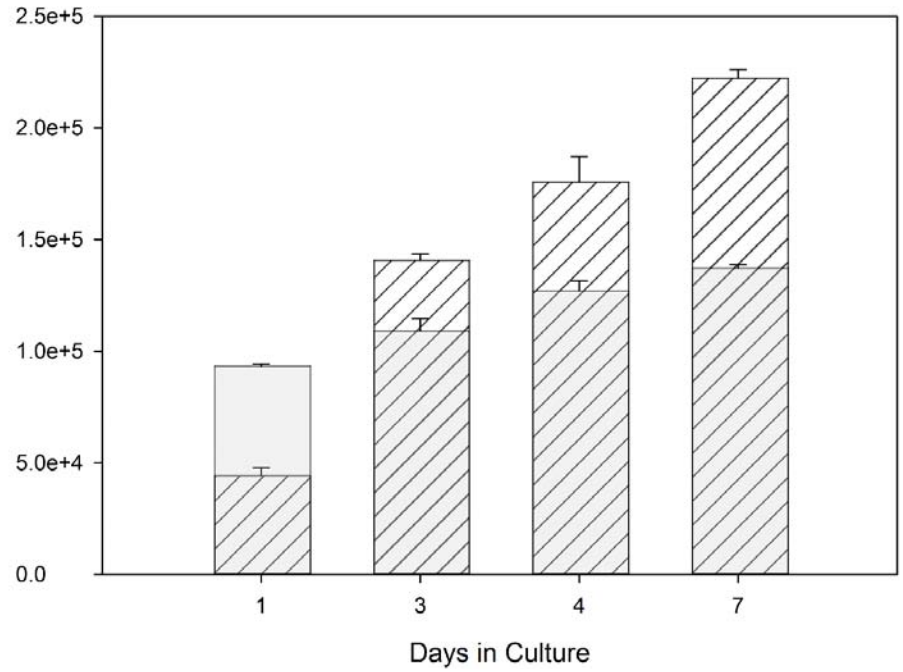

Figure 6. Secretion of a Netrin-3-like protein increases as a function of cell count in *Tetrahymena.* Levels of netrin-3-like protein were taken from conditioned media from cultures that were 1, 3, 4, and 7 days old. Cell counts (diagonal lines) were determined using a BIO-Rad TI-80 cell counter. Fluorescence units (gray bars) were calculated by ELISA, using an antibody directed against the N-terminal peptide of human netrin-3. Each bar represents the mean ± standard deviation of three measurements.

Table 4. Pharmacological inhibition assays suggest that netrin-1-peptide and netrin-3-peptides signal through different pathways in *Tetrahymena thermophila*. Data from our current study were compared with our previous work on netrin-1 peptide [15].

Pharmacological inhibitor	Mechanism of action	Inhibits avoidance to Netrin-1-peptide	Inhibits avoidance to Netrin-3-peptides
GDP-β-S	Inhibits G proteins	No	No
EGTA	Chelates extracellular calcium	No	**Yes**
BAPTA-AM	Chelates intracellular calcium	No	**Yes**
Genistein	Tyrosine kinase inhibitor	**Yes**	No
Apigenin	Broad Spectrum kinase inhibitor	**Yes**	**Yes**

As seen in Table 1, cells adapted to N3C showed only baseline avoidance to N3N and vice versa. In contrast, cells that were adapted to netrin-3 peptides showed typical avoidance when exposed to 1 μM netrin-1-peptide, and cells adapted to netrin-1-peptide showed typical avoidance when exposed to netrin-3 peptides. In vertebrate systems, differences between netrin-1 and netrin-3 signaling are also seen. In vertebrate systems, netrin-3 binds to both deleted in colorectal cancer (DCC) and the UNC-5 family of receptors; however netrin-3 has a lower affinity for DCC than does netrin-1 [11, 20].

Our pharmacological inhibition assays (Figures 2 and 3) showed that N3N chemorepellent signaling is dependent on calcium and serine/threonine kinase activity, since calcium chelators and serine/threonine kinase inhibitors both effectively eliminated avoidance to N3N, reducing it to baseline levels [18, 19]. In contrast, netrin-1 signaling in *Tetrahymena* is calcium independent and involves tyrosine kinase activity [15]. The fact that calcium chelators fail to block signaling through netrin-1-peptide indicates that these drugs are not simply causing a general loss of avoidance in *Tetrahymena.* Consistent with our apigenin data, further investigation using ELISA (Table 2) and immunofluorescence (Figure 4) confirmed that netrin-3-peptides significantly increase serine phosphorylation in *Tetrahymena*.

Through growth assays and viability testing, we established that N3N significantly inhibits mitosis in *Tetrahymena* at a concentration of 1 µg/ml (Figure 5), which was the EC_{100} of this compound in behavioral studies (Figure 1). Our growth assays suggest that calcium and serine/threonine kinase signaling are involved in N3N-mediated mitotic inhibition, as they are in chemorepellent signaling, since calcium chelators and kinase inhibitors both were able to increase the cell count to near-control levels (Figure 5). Cells treated with EGTA did not completely recover from N3N treatment; however, we did not increase the concentration of EGTA in the study because, while doing behavioral assays, we have observed that prolonged exposure to 100 µM EGTA is sometimes toxic to *Tetrahymena.* Nonetheless, the rescue of cells with 50 µM EGTA is significant, and is consistent with a role for calcium in N3N-mediated mitotic inhibition.

We wanted to determine whether N3N affects progression through the cell cycle in addition to affecting cell count. Our DAPI data show that the mean nuclear radius of cells treated with N3N is significantly larger than untreated controls, suggesting that more N3N-treated cells are in G_2 of the cell cycle when compared to untreated controls (Table 3). This may be an indication that cells arrest in G_2 after being exposed to N3N.

Since *Tetrahymena* respond behaviorally and mitotically to N3N and N3C, we hypothesized that *Tetrahymena* may secrete a netrin-3-like protein. As demonstrated by ELISA, secretion of a netrin-3-like protein increases with culture age and cell number (Figure 6). These data are consistent with the hypothesis that that *Tetrahymena* secrete a netrin-3-like protein for intercellular communication to prevent local populations of *Tetrahymena* from depleting available resources. Theoretically, this could occur both by repelling nearby cells, causing them to spread out, and by inhibiting mitosis, thus conserving available energy.

Finally, we compared our recent data on netrin-3-peptides with our previously published data on netrin-1-peptides. Although some studies in vertebrates have found netrin-1 and netrin-3 to be "functionally equivalent" [21] we found vast differences in signaling between the two types of peptides in *Tetrahymena*. First, we found that N3N and N3C do not exhibit cross adaptation with netrin-1-peptide (Table 1). Pharmacological inhibition assays reveal additional differences (Figure 2, 3). Netrin-1-peptide appears to signal through tyrosine kinases [15], while netrin-3-peptides appear to signal through serine phosphorylation (Figure 3, 4; Table 2). Signaling through netrin-3-peptides is dependent on calcium, as is the case with many chemorepellents in *Tetrahymena* [18, 19, 22], but signaling through netrin-1-peptide in *Tetrahymena* appears to be calcium-independent [15]. Secondly, only netrin-3-peptides appear to affect mitosis in *Tetrahymena*; when netrin-1 is added to a culture of *Tetrahymena*, the concentration of cells is not significantly different from the control concentration (unpublished data).

CONCLUSION

In conclusion, our current work has shown differences between the signaling pathways and physiological effects of netrin-3 and -1-peptides in *Tetrahymena*, even though both function as chemorepellents. In particular, we found that netrin-3-peptides serve as mitotic inhibitors in *Tetrahymena*, while netrin-1-peptide does not. If peptides derived from these two netrins have such different effects in *Tetrahymena,* it is possible that they could differentially affect cell division in animal systems. In view of these data, and since roles for netrin-1 in cancer continue to be investigated [13, 21, 23, 24], it may be worth

further study in animal models to investigate whether netrin-3 plays a role in mitotic regulation.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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