Published in final edited form as:

Chembiochem. 2013 February 11; 14(3): 307–310. doi:10.1002/cbic.201200712.

Small-Molecule-Mediated Axonal Branching in *Caenorhabditis elegans*

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Keywords

Caenorhabditis elegans; green fluorescent protein; neuronal branching; small molecules; structure–activity relationships

The complex synaptic connectivity of neuronal circuits enables our abilities to apprehend our environment, to act, and to remember. The formation of these neuronal networks both during development and in adulthood is coordinated through axonal branching, which permits a single neuron to relay information through its axon to multiple coupled neurons. This process is highly regulated and leads to the targeting and plasticity of neuronal circuits. The formation of branches from the axonal shaft is initiated by the protrusion of filopodia or lamellipodia that require subsequent stabilization,^[1–3] as the dynamic nature of the newly formed branch allows either continued growth or retraction back into the axon.^[4–6] The initiation and stabilization of branches can lead to the formation of new, functional neuronal circuits. Agents that can promote post-developmental axonal branching hold promise in the discovery of treatments for disorders caused by defects in connectivity that occur during development. Additionally, the induction of branching can promote the regeneration of axonal connections that have been damaged due to disease or injury.^[7]

The majority of screens to identify small molecules that are capable of promoting the survival and growth of neurons employ in vitro systems that utilize cell lines or primary neuronal cultures. These assays, although straightforward, fail to reflect the conditions of networked neurons and do not account for metabolism, drug access to specific tissues, or toxicity. To develop a straightforward, easily implemented assay that reflects the outgrowth of networked neurons in vivo, we have established a new protocol utilizing the nematode *Caenorhabditis elegans*. The complete mapping of the roundworm's 302 neurons makes the organism ideal for these studies.^[8] A number of important conserved biological functions, such as apoptosis, have been elucidated through studies using *C. elegans*.^[9] Additionally,

bioinformatic analysis estimates that 60–80% of the genes of the worm are homologous to human genes.^[10] The transparent nature and facile incorporation of green fluorescent protein (GFP) greatly facilitate the imaging of neuronal morphology in live animals, and readily permit the visualization of axonal branching.^[11] The organism's low cost, short developmental cycles, and ease of use makes it ideal for high-content screening.^[12, 13]

Small-molecule-mediated axonal branching was discovered by treating *C. elegans* having GFP labeling of different neuronal classes with a known promoter of neuronal growth, clovanemagnolol (**1**), and monitoring the animals for aberrant, additional neuronal growth (Figure 1).^[14, 15] In addition to developing a laboratory synthesis of clovanemagnolol based on biosynthetic consideration,^[16] we have found that it is a potent neurotrophic agent that, at concentrations of 10 nm, induces the outgrowth of both hippocampal and cortical neurons.^[17, 18] After a screen of the different neuronal classes, it was found that *C. elegans* strain LX929 (vsIs48[unc-17: :GFP]),^[19] which expresses GFP in all cholinergic neurons, displayed axonal branching in 35% of the worms treated with clovanemagnolol (**1**). Some branching was also evident in the ALM of worms with GFP-labeled glutamatergic touch neurons, although it was not as pronounced, with only slight projections extending past the cell body and no axonal branching as present in the cholinergic system. We therefore focused on the cholinergic strain. Due to the complex nature of cholinergic nerves fluorescing in the nerve ring, examinations for branching focus on regions posterior to the pharynx. Interestingly, control worms have a lower, consistent rate of background branching in their cholinergic neurons, a previously unreported phenomenon.

Physical stress did not increase branching in untreated control or small-molecule-treated worms. There was no evidence of severed or otherwise injured axons, and the background rate of branching was unaffected by changes in the conditioning of the worms. Untreated control worms showed a consistent rate (18%) of background branching when subjected to agitation by vortexing or excessive prodding during the picking of L4 larvae, similar to when physical stress was not applied ($n = 40$). Similarly, worms treated with compound showed the same amount of increased branching in both physically stressed and unstressed

worms ($n = 40$). Likewise the age of the worms (up to one-week-old adults) did not affect the rate of branching, as seen in mechanosensory and GABAergic neurons, where the rates are influenced by Jun kinase and the insulin/IGF-1 pathways, which affect the general rates of aging.^[20] However, increasing amounts of branching were seen with additional aging past one week, and at 10 days, half of untreated control worms ($n = 20$) possess abnormal branching morphologies. As a result, the studies examining the effects of the compounds upon branching were confined to late larval stages and early adulthood.

We examined worms ($n = 20$) at several time points over 3 days following treatment with small molecules. Outgrowth could be observed by 24 h after treatment, and changes such as partially shortened branches or additional and lengthened branches were present in the same animal at 30 h. These observations are consistent with the formation and retraction of branches within *C. elegans* functioning as a dynamic process that is enhanced by small molecules. The overall percentage of outgrowth was similar by 48 h after treatment. No significant changes were observed by extending exposure time to 72 h, thus we limited our experiments to 48 h.

To ensure that the *Escherichia coli* food source did not interfere by metabolizing the applied small molecules prior to absorption by *C. elegans*, experiments were conducted on both live and heat-killed bacteria. Outgrowth was observed in 33% of worms ($n = 40$) exposed to clovanebisphenol and 35% of worms ($n = 40$) exposed to the natural product vinaxanthone on plates with dead bacteria, compared to 30 ± 5 and $31 \pm 7\%$, respectively, with live *E. coli*.

Branches that were observed upon treatment with clovanemagnolol included single processes and Y-shaped growths as well as other aberrant growth patterns (Figure 1). Most significantly new branching was observed to originate from the dorsal nerve cord, the ventral nerve cord, and sublateral nerve cords, with growth occurring toward the opposite side of the worm. No pattern in the location of this branching was observed as it originated from the dorsal and ventral sides with approximately equal frequency. Wandering commissural growth and branching from commissures that run ventrodorsally across the body of the worm were also observed, but less commonly. Branching, both single process and occasionally Y-shaped growth, was observed from the ventral and dorsal nerve cords as well as sublateral nerve cords. Single branches were the most commonly observed growth morphology, although multiple branches were frequently seen in treated worms. This complex outgrowth was never present in control worms. Similar outgrowth effects were present following treatment with the natural product caryolanemagnolol (**2**), which possesses comparable activity in promoting branching in primary neuronal cultures. The bis-allyl phenol fragment of **1** and **2**, magnolol (**3**), did not induce outgrowth, and is likely quickly metabolized.

With this straightforward assay for detecting neurotrophic compounds, a hand-curated small-molecule library of commercially available growth-promoting compounds as well as compounds synthesized in our laboratory was screened to discover small molecules that can promote branching in vivo. The compounds were designated as promoting outgrowth if 25% of the treated worms possessed branching. Although the accumulation of compounds

in *C. elegans* has been reported as challenging,^[21] we found that several compounds, mostly natural products, possessed activity and resulted in the outgrowth phenotype (Figure 2).

Among the compounds screened (see Table S1 in the Supporting Information), several structurally diverse molecules were shown through the in vivo assay to significantly promote outgrowth (Figure 3). Dibutyryl-cAMP (**4**) caused branching to occur in 36% of the treated worms. This cell-permeable derivative of cAMP stimulates cAMP-dependent protein kinase and upregulates Nur77 expression through histone modification, thus promoting outgrowth in a NGF-independent manner.^[22, 23] Carnosic acid (**5**) was found to induced branching in 36% of the worms. Carnosic acid was previously shown to enhance the synthesis of nerve growth factor (NGF) in human glioblastoma cells and of brain-derived neurotrophic factor (BDNF) in dopaminergic neuronal cell lines;^[24, 25] this effect has been reproduced in vivo through intranasal delivery of carnosic acid in rats.^[26] However, as *C. elegans* do not have genes that encode for neurotrophins or neurotrophin receptors, here alternative growth-promoting pathways must be activated by carnosic acid. The polyprenylated benzophenone natural product garcinol (**6**) led to outgrowth in 39% of worms. Garcinol also promotes the growth of rat cortical progenitor cells through the activation of extracellular signal-regulated kinase (ERK) and enhances neuronal survival under conditions of growth factor deprivation.^[27] Trigo-nelline (**7**), a natural product isolated from coffee beans, also increased outgrowth, causing branching in 28% of the worms. This nicotinic acid derivative also enhanced the number of human neuroblastoma SK-N-SH cells that have neuritic growth.^[28] The anti-fungal agent amphotericin B (**8**) promoted outgrowth in 36% of treated animals. Previous studies of amphotericin have shown that the natural product can promote neurite outgrowth over a wide range of myelin-associated inhibitors and chondroitin sulfate proteoglycans through the activation of AKT.^[29]

In addition to identifying neurotrophic compounds, the assay enabled medicinal-chemistry-based efforts to edit the structure of clovanemagnolol. Through the use of an existing synthetic route to clovanemagnolol,^[16] analogues were prepared and tested for their ability to promote outgrowth. After several derivatives had been tested, the doubly des-allyl compound clovanebisphenol (**11**) was found to promote outgrowth in an analogous manner to clovanemagnolol, with 30% of worms displaying branching (see Figure S1). A simplified one-step synthesis of this compound from commercially available material has been developed (Scheme 1 and the Supporting Information), thus allowing easy access to gram-scale quantities for future studies.

These studies have shown the broad utility of our new in vivo outgrowth assay in *C. elegans* to enable the identification of compounds that can promote branching. The growth-promoting compounds detected in the screen included amphotericin B, trigonelline, dibutyryl-cAMP, carnosic acid, and garcinol. In addition, this assay could be used to guide the biological optimization of small molecules. Chemical editing of clovanemagnolol led to the discovery of the des-allyl compound clovanebisphenol, a compound with a simplified structure and equal potency.

Experimental Section

Nematode cultures

C. elegans were maintained on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 bacteria at 22 °C according to standard procedures.^[30] Strains used include LX929 (vsIs48[unc-17: :GFP]), EG1285 (oxIs12 [Punc-47: :GFP; lin-15(+)]), BZ555 (egIs1[Pdat-1: :GFP]), SK4005 (zdIs5[Pmec-4: :GFP; lin-15(+)]), and LX959 (vsIs1 3[lin-11 : :pes-10: :GFP; lin-15(+)]). Nematode strains were obtained from the Caenorhabditis Genetics Center, which is funded by the NIH Office of Research Infrastructure Programs.

Outgrowth assays

Stock solutions (20 mM) of various chemicals were prepared in DMSO and subsequently diluted in M9 buffer^[12] to a final concentration of 0.04 mM (0.2% DMSO, v/v). Diluted solutions (200 µL) were spread over seeded NGM plates (35 × 10 mm, containing 4 L of agar) and allowed to absorb to a final concentration of 2 µM. Approximately 30 fourth-larval-stage nematodes were picked from age-synchronized populations to prepared chemical plates. Worms were allowed to grow at 22 °C on the chemical plates for approximately 48 h, after which time the day-2 adults ($n = 20$) were mounted in M9 buffer (10 µL) on 2% agarose pads containing sodium azide (5 mM) and observed for neuronal outgrowth compared to untreated control worms. All experiments were repeated in triplicate.

Microscopy

An Olympus SZX16 stereomicroscope was used to manipulate *C. elegans*. GFP-labeled nematodes from outgrowth assays were visualized at 400× magnification by using an Olympus IX51 inverted microscope with an X-Cite 120Q fluorescence microscopy illumination system. Images were captured on a QImaging Retiga-2000R Fast 1394 Mono digital camera.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support from The University of Texas at Austin, the Welch Foundation (F-1694), and the Norman Hackerman Advanced Research Program (003658-0034-2011) is gratefully acknowledged.

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Figure 1. Cholinergic branching in *C. elegans*. A), B) Normal morphology in the ventral and dorsal nerve cords and commissures. C), D) Lower level background branching observed in untreated control worms. E), F) Branching caused by the natural product clovanemagnolol (**1**). G), H) Complex branching in animals treated with caryolanemagnolol (**2**).

Figure 2. Representative outgrowth induced by small molecules. Nematodes treated with A) amphotericin B, B) trigonelline, C) dibutyryl-cAMP, D) carnosic acid, and E) garcinol.

Figure 3.

A) Outgrowth reported for nematodes treated with 2 μ M small molecules, with branching observed in 25% of nematodes. B) The structures of the active compounds identified above.

Scheme 1.

One-step synthesis of clovanebisphenol from β -caryophyllene oxide. a) $(\text{PhO})_2\text{P}(\text{O})\text{OH}$, CH_2Cl_2 , 23°C , 16%.