Bacterial Neurotoxicity and Parkinson's Disease

Jeana Blalock and Jafa Armagost
Dr. Guy Caldwell, Ph.D, Dr. Kim Caldwell, Ph.D.
Professors of Biological Sciences, The University of Alabama

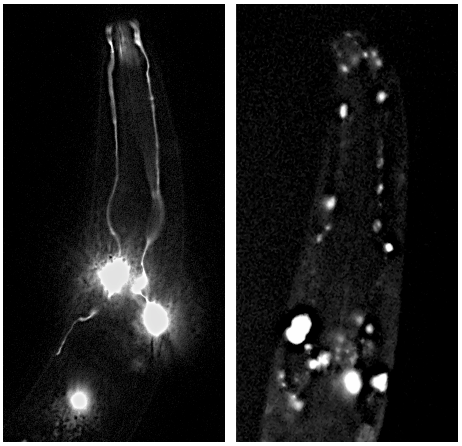
Parkinson's disease (PD) involves the progressive loss of dopamine (DA) neurons from the substantia nigra pars compacta. Genetic forms of PD account for only 5-10% of known cases and environmental factors appear pivotal to sporadic causality. Evidence from both environmental and genetic forms of PD indicates that overloading the ubiquitin-proteasome system is a causative risk factor for PD. Furthermore, when proteasome inhibitors were injected directly into the brains of rats, PD-like symptoms resulted (McNaught et al., 2004; Ann Neurol. 56:149-162). Many proteasome inhibitors were originally isolated from bacterial strains within the order Actinomycetales. There is a high rate of sporadic PD among patients from rural backgrounds, where well water and farming potentially represent sources of increased exposure to Actinomycetes. We determined that exposure to Actinomycetes caused DA neurodegeneration in C. elegans expressing a GFP reporter in DA neurons (dat-1::GFP). Interestingly, when dat-1::GFP worms were grown on S. lividans and S. venezuelae, they displayed DA neurodegeneration that increased over time, where at 4 days exposure 52% and 59% of DA neurons were abnormal, respectively, compared to 19% of worms grown on E. coli OP50.

Introduction

Parkinson's disease (PD) is a human movement disorder distinguished by bradykinesia (slowness of movement), rigidity, and tremors. As the second most common neurodegenerative disease, PD is characterized by the loss of dopamine (DA) neurons. The environment may have a key role in 90%-95% of PD as only 5%-10% of cases are known to have genetic causes. Parkinson's disease has been correlated to living a rural lifestyle. People who work with the soil and drink well water have a higher prevalence of PD (1). Some known environmental factors include pesticides (rotenone), herbicides (paraquat), and heavy metals (manganese) (1). The neurotoxin BMAA, which was isolated from cyanobacteria, caused a high incidence of neurological disorders, including PD, in Guam (2).

Bacterial infections may also be related to PD. Antibodies to *Nocardia asteroides* have been found in the blood serum of PD patients (3). *N. asteroides*, an Actinomycete, is also known to cross the blood brain barrier. Many Actinomycetes produce secondary metabolites, such as antibiotics and proteasome inhibitors. McNaught and coworkers showed that the injection of proteasome inhibitors into the brains of rats caused PD-like symptoms (4). These results are interesting since the ubiquitin proteosome system (UPS) in PD patients is often faulty. The UPS breaks down proteins within cells and is important for cellular function (5). Some Actinomycetes are pathogenic, such as *N. asteroides*. Others, such as species of *Streptomyces*, are commonly found in the soil. These non-pathogenic Actinomycetes can also produce secondary metabolites, including proteosome inhibitors.

The soil contains a variety of components including fungi, bacteria, and nematodes (worms). *Caenorhabditis elegans* are one such nematode. *C. elegans* were the first multicellular organism to have its entire genome sequenced and have been used as a model organism since 1965. As a model organism, *C. elegans* is ideal for looking at the relationship between bacteria and PD because worms eat bacteria (*Escherichia coli* strain OP50). A short life span of 14-17 days and short generation time of 3 days enables researchers to look at numerous worms quickly. *C. elegans* have a simplified nervous system composed of 302 neurons compared to the billions of neurons in humans (6). Additionally, the nervous system of *C. elegans* has been completely mapped. *C. elegans* have 8 dopamine neurons (6 in the head and 2 in the tail) that are lit up with green fluorescent protein (GFP), which was originally isolated from jelly fish (6, 7). In order to visualize the neurons in the worms, GFP was cloned along with the promoter and inserted into the genome of the worm resulting in neurons that express GFP.



OP50 *S. lividans**

* except for DA neuronal degeneration, worms exposed to these *Streptomyces* appeared healthy

The above images show an example of the neurons observed in *C. elegans* with regards to their bacterial exposure. The image on the left labeled "OP50" shows wild type neurons (normal, typical). The image on the right labeled "S. lividans" displays DA neurons after three days of exposure to a specific strain of *Streptomyces*. The degeneration of DA neurons is characteristic of the onset of PD.

To examine the potential impact of Streptomyces exposure on worm dopamine (DA) neurons, this study characterized the effect of *Streptomyces lividans, Streptomyces venezuelae, Streptomyces coelicolor,* and *Streptomyces griseus* on *C. elegans* DA neurons.

Materials and Methods

Direct Exposure to Streptomyces Bacterium

Caenorhabditis elegans were exposed to a 7:1 ratio of *Streptomyces* (either *S. lividans, S. venezuelae, S. coelicolor, or S. griseus*) and *E. coli* on nematode growth medium (NGM). The NGM plates were inoculated with 100µL of the *Streptomyces*/*E. coli* mixture and allowed to grow into a confluent lawn by incubating for 2 days at 20°C. Between 50 and 100 juvenile $P_{dat\text{-}1}$::GFP worms (L4 stage) were transferred onto the bacterial lawns. The worms were then transferred to plates containing freshly grown *Streptomyces*/*E. coli* every other day to ensure the original generation was analyzed. Approximately 30 worms were analyzed for DA neuron degeneration every day for 4 consecutive days of exposure to the *Stretomycetes*. Species resulting in a positive result were then analyzed for an additional 3 days, for a total of 7 days of analysis. Each experiment was done in triplicate.

Dopamine Specificity

Caenorhabditis elegans, containing GFP expressed in GABAergic and cholinergic neurons ($P_{unc\text{-}4}$::GFP and $P_{unc\text{-}47}$::GFP respectively), were analyzed for neuron degeneration in order to determine if the degeneration was specific to DA neurons. The analysis was done after 4 days of exposure to *S. lividans* and *S. venezuelae.*

Conditioned Media

Bacterial Growth and Extraction

Conditioned media was made by growing *S. lividans, S. venezuelae, S. coelicolor, S. griseus,* and *E. coli (OP50)* in SYZ media. Approximately 1200 mL of SYZ media containing 1 x10^6 spores/mL were grown at 30°C for 162-168 hrs in fernbach flasks. The cultures were then centrifuged to pellet the majority of the cells. The supernatants from the centrifugation were filter sterilized using 0.22µm Polyether sulfone (PES) filters to remove any remaining cells. The excretions were then stored at -20°C until use.

Worm Exposure to Streptomyces Excretions

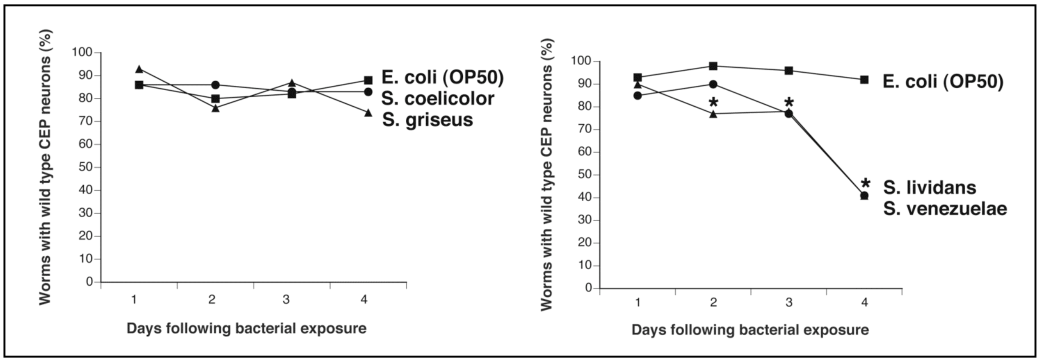
The conditioned media was spread onto NGM plates (25µL/mL) where it was allowed to soak into the agar (~24 hrs). 400µL of concentrated *E. coli* culture was then added to create a bacterial lawn. Approximately 50-100 juvenile (L4) $P_{dat\text{-}1}$::GFP worms were transferred to the conditioned plates. The worms were transferred to fresh plates containing excretions every 2 days to ensure the original generation of worms was analyzed. For each day of exposure 30 worms were analyzed per condition. Each experiment was done 6 times for a total of 180 worms analyzed per condition.

Results

Direct Exposure to Streptomyces Bacterium

This study looked specifically for degeneration caused by Streptomyces in the 4 most anterior DA neurons (CEP) in *C. elegans.* S. *coelicolor* and *S. griseus* did not show significant DA neuron degeneration compared to the *E. coli* as the percent of worms with completely wildtype neurons was 82%, 70%, and 90%, respectively (see Figure 1, left). However, after 4 days of exposure to *S. venezuelae* and *S. lividans*, only 41% and 48% of

Figure 1. Percentage of wildtype worm neurons following four days of bacterial exposure.



the worms displayed wild-type neurons, respectively. Exposure to these two species of bacteria was continued through 7 days, as shown below (see Figure 1, right).

Dopamine Specificity

To determine if the effect was specific for DA neurons, or if other neuron classes such as GABAergic or cholinergic neurons were affected by the exposures, we examined these neuron classes as well. We determined that after 4 days of exposure to *E. coli, S. lividans* or *S. venezuelae,* neither GABAergic nor cholinergic neurons were affected (see Table 1). However, DA neurons showed 50% degeneration after exposure to both *S. lividans* and *S. venezuelae.* In PD the first neurons to show degeneration are DA neurons. The susceptibility of DA neurons in PD as well as this study, suggest that these bacterium may play a role in PD.

Table 1. Neuron degeneration following exposure to *Streptomyces* species results in DA neuron degeneration, not cholinergic or GABAergic degeneration. Values displayed are standardized compared to the control, *E. coli*.

Bacterial Exposure	cholinergic	GABA	DA
E. coli	N/A (n=51)	N/A (n=47)	N/A (n=50)
S. lividans	97% (n=50)	100% (n=45)	50% (n=50)
S. venezuelae	97% (n=49)	98% (n=52)	50% (n=50)

Conditioned Media

As *Streptomyces* are known to secrete secondary metabolites, we wanted to determine if the neurodegenerative factor is secreted. To test this, all four species of *Streptomyces*, as well as *E. coli*, were grown in liquid media for 7 days, and then the cells were removed by centrifugation and filtration. Equivalent amounts of liquid suspension were then placed on agar plates for worm exposure. Our analysis showed that the factor responsible for the DA neuron degeneration was excreted from *S. lividans* and *S. venezuelae*. As shown in the figure below, both *S. lividans* and *S. venezuelae* showed statistically significant DA neuron degeneration 4 days after exposure while *E. coli* exhibited no DA neuronal degeneration. The DA neuronal degeneration continued and increased over the

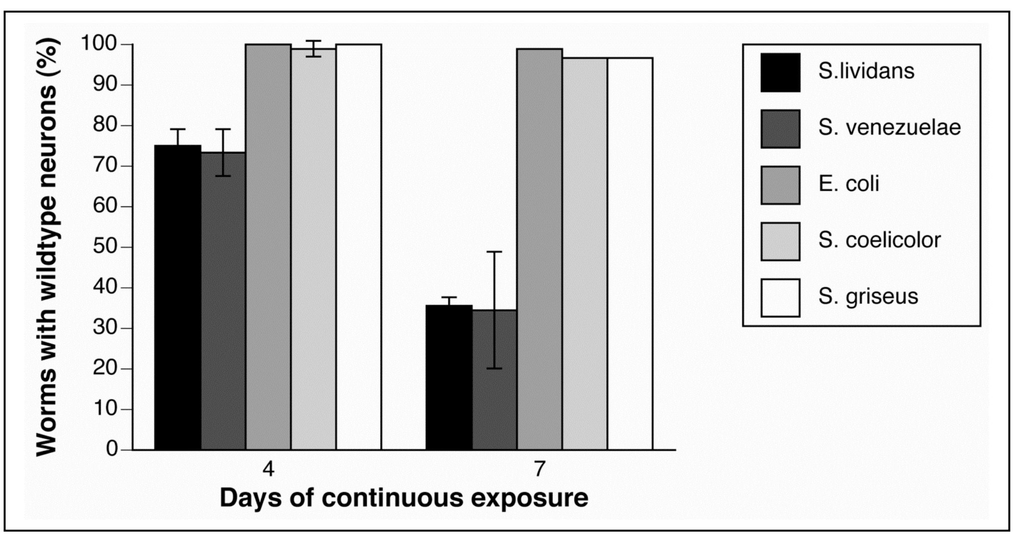


Figure 2. Graphical representation of wildtype worm neurons following exposure to bacterial excretions

7 day time period. Notably, neither *S. coelicolor* nor *S. griseus* showed significant DA neuron degeneration at either time point (see Figure 2).

Discussion

These data demonstrate that *C. elegans* DA neurons undergo degeneration following exposure to an excreted product produced by *Streptomyces lividans* and *Streptomyces venezuelae.* Taken together with high prevalence of PD found in people who work in the soil or drink well water, these patients may be exposed to factors excreted by soil bacteria that have been shown to cause DA neuron degeneration in *C. elegans.*

References

1. Lai B.C.L., Marion S.A., Teschke K., Tsui J.K.C. (2002) Occupational and environmental risk factors for Parkinson's disease. *Parkinsonism & Related Disorders.* 8(5), 297-309.
2. Cox P.A., Banack S.A., Murch S.J., Rasnussen U., Tien G., Bidigare R.R., Metcalf J.S., Morrison L.F., Codd G.A., Bergman B. (2005) Diverse taxa of cyanobacteria produce beta-N-methylamino-L-alanine, a neurotoxic amino acid. *Proc Natl Acad Sci USA*. 102, 5074-8.
3. Hubble J.P., Cao T, Kjelstrom J.A., Koller W.C., Beaman B.L. (1995) Nocardia species as an etiologic agent in Parkinson's disease: serological testing in a case-control study. *J Clin Microbiol*. 3(10), 2768-9.
4. McNaught K.S., Perl D.P., Brownell A.L., Olanow C.W. (2004) Systemic exposure to proteasome inhibitors causes a progressive meodel of Parkinson's disease. *Ann Neurol*. 56, 149-62.
5. Tanaka K. (2006) Impairment of the ubiquitin-proteasome system and neurodegeneration. *Nihon Shinkei Seishin Yakurigaku Zasshi.* 26(2), 67-73.
6. Eisenmann, D. M., Wnt signaling (June 25, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.7.1, http://www.wormbook.org.
7. Chalfie M., Yuan Tu, Euskirchen G., Ward W.W., Prasher D.C. (1994) Green Fluorescent Protein as a Marker for Gene Expression. Science. 263, 802-805

Acknowledgements

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Jeana Blalock, a junior biology major from Black, Alabama, is an active member of the University Honors Program, Phi Sigma Pi and Tri-beta. She began studying biology at UA as a Howard Hughes Rural Science Scholar and currently conducts research in the Caldwell lab as an undergraduate researcher in the Howard Hughes Medical Institute Internship Program.