

Hypothesis-based Screening of Candidate miRNAs in Dopaminergic Neuroprotection

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Abstract

*MicroRNAs (miRNAs) are single-stranded molecules of RNA about 20-23 nucleotides in length that act in regulation of gene expression. Our laboratory has conducted a bioinformatic analysis that uncovered certain miRNA molecules that may play a role in the regulation of multiple neuroprotective genes. Age-dependent dopamine neuron degeneration is a hallmark of Parkinson's disease (PD); thus, factors that may operate in pathways leading to this result warrant further investigation. Utilizing the nematode *Caenorhabditis elegans* as a model system for studying PD, we have previously identified a set of genes that confer neuroprotective capacity against insults linked to PD in humans, notably the overproduction of a protein termed α -synuclein. Here we experimentally examine the hypothesis that select miRNAs may function to co-regulate target genes in networks that can attenuate α -synuclein toxicity and cell death. We are conducting a series of genetic crosses between mutant worms lacking specific*

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*miRNA function with transgenic nematodes overexpressing human α -synuclein in dopamine neurons. This strategy enables rapid validation of bioinformatic predictions of miRNA targets by scoring for the impact of selected miRNAs on neurodegeneration over the course of the short two-week lifespan of *C. elegans*. In this manner, the identification of specific regulatory miRNAs that function to mediate neuronal survival would represent novel targets for therapeutic development.*

Parkinson's disease (PD) is a movement disorder of the central nervous system that affects over 1.5 million Americans. Approximately 10% of all cases of the disease are determined to be genetic while the other 90% are estimated to be the result of an interplay between environmental and genetic factors.

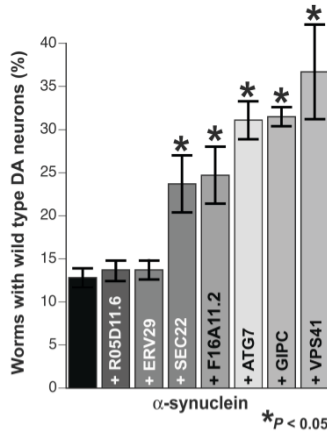
Defining characteristics of PD include age-dependent loss of dopamine (DA) neurons within the *substantia nigra pars compacta* as well as the formation of Lewy bodies within the DA neurons (1). Lewy bodies are large inclusions centrally composed of an aggregated protein called α -synuclein (α -syn) which, in this aggregated state of insoluble fibrils, leads to the death of DA neurons. Both multiple copies (referred to as "overexpression") of wild type human α -syn under the DA promoter, as well as mutant forms, have been shown to cause dopaminergic neurodegeneration within the *Caenorhabditis elegans* model, this giving the organism an observable Parkinson's-like phenotype (2).

The nematode *C. elegans* serves as a key PD model due to its prime ability to be genetically manipulated. Not only does *C. elegans* cost very little to maintain, but the generation of progeny is a relatively quick process. The structure of the transparent nematode also allows for simple *in vivo* functional analysis (3). For example, a worm strain overexpressing human α -syn fused to GFP, the Green Fluorescent Protein, in muscle cells was used to systematically screen nearly 900 genetic candidates, chosen due to their relation to known PD genes or their link to aggregate formation, i.e., protein misfolding and degradation pathways (4). This screen was performed using RNA-interference (RNAi), which knocks down the expression of specific genes. Interestingly, five genes identified from the screen, which have not been linked to PD previously, have been demonstrated to ameliorate α -syn-induced neurotoxicity in the worm's DA neurons (5).

With the aim in mind of discerning protective strategies against age-dependent neurodegeneration generated by human α -syn, newer mechanisms

involving this most recent published data warrant further investigation, including the exploration of synergies within networks of neuroprotective genes. Select experiments can then be used to elucidate the cellular pathways associated with neurodegeneration. Genes that demonstrate notably significant neuroprotection against α -syn toxicity, including those shown in the published data in **Figure 1**, are able to be grouped according to their cellular functions and roles in maintaining homeostasis within the cells (5).

Fig 1. Graph depicting the percentage of worms expressing α -syn with wild type (WT) DA neurons when candidate neuroprotective genes are co-expressed. The control α -syn line is shown in black at the far left.

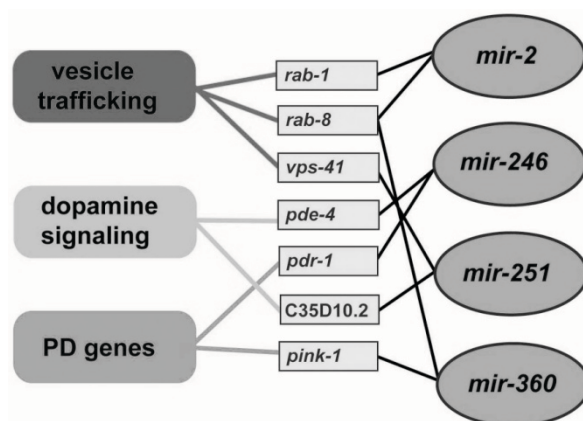


Using bioinformatic analysis on these select genes targets, our laboratory has mined the database *miRBase*, of the Sanger Institute, in order to acquire evidence of potential interconnectivity in the *C. elegans* genome. Through this data-mining, several distinct relationships emerge that represent ideal targets for functional validation of microRNA co-regulation.

MicroRNAs (miRNAs) are endogenous single-stranded molecules of RNA, generally about 21-23 nucleotides in length. They play important roles in global gene regulation in animals and plants at the posttranscriptional level via affecting specific messenger RNA (mRNA) targets for cleavage or translational repression. Though the mechanisms behind the processing of miRNAs are not yet completely understood, it is known that there are hundreds of miRNAs present within the organism, all very highly conserved throughout species. These hundreds of miRNAs are responsible for the regulation of thousands of genes (6, 10). The hypothesis then surfaces that a loss-of-function mutation in a certain miRNA would prevent its down-regulation of the target gene, and therefore would allow the gene product in question to function unchecked.

The information collected from *miRBase* resulted in four lead miRNAs being uncovered. These include mir-2, mir-246, mir-251, and mir-360. Each of these potentially regulates more than one neuroprotective gene (*rab-1/rab-8*, *pde-4/pdr-1*, *C35D10.2/vps-41*, *rab-8/pink-1*, respectively). Genes *rab-1* and *rab-8*, as well as *VPS-41*, function in vesicle-trafficking, to which DA neurons are highly sensitive (7, 8). Other gene functions, as well as their predicted targeting miRNAs, are illustrated below in **Figure 2**.

Fig 2. Summary of the connectivity of *C. elegans* miRNAs and their predicted gene target. Cellular function of the genes is listed to the far left, select neuroprotective genes are listed in the center, and the regulatory miRNAs are listed to the right.



Methodology

In order to perform this assay, strains of worms that contain a constitutive overexpression of both α -syn and a mutant miRNA were generated via genetic crosses. Doing so involved mating male worms overexpressing the α -syn gene in the dopamine-transport-driven promoter and GFP under the dopamine transport-driven promoter ($P_{dat-1}::\alpha$ -syn; $P_{dat-1}::GFP$) with hermaphrodites expressing the desired miRNA loss-of-function mutation. Polymerase chain-reactions (PCRs) were run in verification of the presence of homozygosity of the desired deletion mutations within the organism. A total of three outcrosses were performed on each of the transgenic lines in order to ensure the absence of other, undesired mutations in the strains.

These mutation strains include:

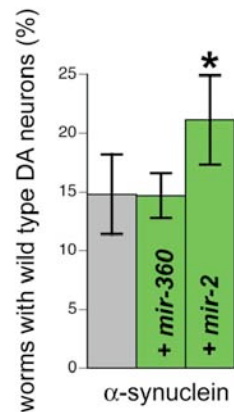
1. mir-2 (allele gk259)
2. mir-360 (allele n4635)
3. mir-246 (allele n4636)
4. mir-249 (allele n4983)
5. mir-251 (allele n4606)

Once these transgenic lines were created, a neurodegenerative assay was performed. The animals were grown to 7-day-old adults and mounted on slides to be observed under a fluorescent filter in the Nikon Eclipse E800 high-powered microscope. Through this method, potential degeneration of the DA neurons can be monitored as GFP will be present in the DA neurons and the neurons show results in classic apoptotic phenotype, including “blebbing,” rounding, and cell death. These phenotypes have been previously developed into an assay form (9). Significant neuroprotectivity was analyzed based upon the number of worms with complete sets of all six anterior DA neurons intact, referred to as wild type (WT).

Results and Discussion

The worm strains were analyzed according to the previously described phenotypes of the DA neurons. A mutant strain demonstrating a decrease in DA neurodegeneration may be potentially enhancing the effect of α -syn neurotoxicity. The most recent results can be seen in **Figure 3**, demonstrating the significant neuroprotectivity of the strain of mutant mir-2 co-expression.

Fig 3. Graph depicting the percentage of worms expressing α -syn with WT DA neurons when candidate neuroprotective microRNAs are co-expressed. The α -syn control is shown in grey at the far left. Whereas no change is seen in the percentages between the α -syn control and the line co-expressing mutant mir-360, the line co-expressing mutant mir-2 demonstrates a significant amount of neuroprotection (Student *t* test).



Thus, a new line of thinking involving the mechanisms behind neurodegeneration can begin to evolve, this time with a greater understanding of the many genetic factors that contribute to protection against cellular insults and new implications of novel regulatory mechanisms in neuron survival. According to the search on the *miRBase* database, the two miRNAs, mir-2 and mir-360, have in common a predicted target site within the mRNA of the gene *rab-8*. The results in **Figure 3** could implicate a number of logical interpretations for the lack of significant neuroprotectivity in the worms. First, it is possible that mir-360 is not a true regulator of *rab-8*, and the predicted target site for mir-360 on the gene is false. However, it may be that *rab-1* is the key neuroprotective gene that requires regulation by multiple miRNAs. The regulation by mir-360 could be insignificant unless performed in concert with mir-2. The effect of mir-360 in the regulation of *pink-1* could possibly be similar, indicating other necessary miRNAs in co-regulation.

In the near future, the other miRNA mutants will be analyzed in the same manner, examining any alterations in the α -syn protein's toxic effect, and their results will be recorded. Any transgenic lines that do not exhibit a discernable change in neurodegeneration will be generated instead via direct microinjection of specific miRNAs encoding regions under the control of DA neuron-specific promoter into α -syn + GFP worms. Data from the original RNAi screen previously described (5) will also be incorporated further into the identification of other candidate miRNAs for neuroprotection.

The substantial role of miRNAs has been demonstrated previously in diverse cellular processes, including apoptosis, developmental timing, and muscle growth (10). The results demonstrated thus far allow for the assumption that networks of miRNAs now could be implicated as very important mediators of neuronal survival.

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